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# Two-Photon Excited Fluorescence Detection Technology in Screening for Methicillin-Resistant *Staphylococcus aureus*

by

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*To Helinä, Karoliina and Eero*

# ABSTRACT

Teppo Stenholm

**Two-Photon Excited Fluorescence Detection Technology in Screening for Methicillin-Resistant *Staphylococcus aureus***

The Department of Internal Medicine, the Department of Medical Microbiology, and the Laboratory of Biophysics, University of Turku, Turku, and the Antimicrobial Resistance Unit, National Institute for Health and Welfare, Turku, Finland

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Control of the world-wide spread of methicillin-resistant *Staphylococcus aureus* (MRSA) has been unsuccessful in most developed countries. A few countries have been able to maintain a low MRSA prevalence, plausibly due to their strict MRSA control policies. Such policies require wide-scale screening of patients with suspected MRSA colonization, in order to nurse the MRSA-positive patients in contact isolation.

The aim of this study was to develop and introduce a 2-photon excited fluorescence detection (TPX) technique for screening of MRSA directly from clinical samples. The assay principle involves specific online immunometric monitoring of *S. aureus* growth under selective antibiotic pressure.

After the novel TPX approach had been set up, its applicability for the detection of MRSA was evaluated using a large MRSA collection including practically all epidemic MRSA strains identified in Finland between 1991 and 2009. The TPX assay was found both sensitive (97.9%) and specific (94.1%) in this epidemiological setting, illustrating that the method is tolerant to wide biological variation as well as to environments with rapidly emerging MRSA strains. When MRSA was screened directly from colonization samples, all patients positive for MRSA by conventional methods were positive also by the TPX assay. The assay capacity was 48 samples per a test run, and the median time required for confirmation of a true-positive screening test result was 3 h 26 min.

Collectively, the findings presented in this thesis suggest that the TPX MRSA screening assay could be applicable for direct screening of MRSA colonization samples without any prior steps of isolation. This can potentially mean that contact isolation of suspected carriers testing negative could be discontinued earlier, thereby reducing the costs and burden associated with the containment of MRSA. In case of infection, a positive test result would ensure an early onset of effective therapy.

**Keywords:** MRSA, TPX, antimicrobial susceptibility testing, high-throughput assay, 2-photon excited fluorescence, point-of-care

# TIIVISTELMÄ

Teppo Stenholm

**Kaksoisfotonivirittеiseen fluoresenssiin perustuva menetelmä metisilliinille resistentin *Staphylococcus aureuksen* seulonnassa**

Sisätautien klinikka ja sisätautioppi, lääketieteellinen mikrobiologia ja immunologia sekä biofysiikan laboratorio, Turun yliopisto, Turku, ja Mikrobilääkeresistenssi-yksikkö, Terveyden ja hyvinvoinnin laitos (THL), Turku

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Metisilliinillle resistentin *Staphylococcus aureuksen* (MRSA) leviämistä ei ole pystytty estämään suurimmassa osassa kehittyneistä maista. Muutamassa maassa MRSA:n esiintyvyys on kuitenkin pysynyt matalana, oletettavasti niissä harjoitettujen tiukkojen sairaalahygieenisten toimenpiteiden ansiosta. Nämä toimenpiteet ovat poikkeuksetta nojanneet laajamittaisiin seulontaohjelmiin ja kolonisoituneiksi epäiltyjen potilaiden hoitamiseen sairaalassa kosketuseristyksessä.

Tämän tutkimuksen tarkoituksena oli kehittää kaksoisfotonivirittеiseen fluoresenssiin (TPX) perustuva uudenlainen menetelmä kliinisten MRSA-näytteiden seulontaan. Menetelmä mahdollistaa *S. aureuksen* kasvun reaaliaikaisen immunometrisen seurannan valikoivassa antibioottipitoisuudessa.

Menetelmän kehitystyön jälkeen tutkittiin laajan biologisen variaation ja muuttuvan epidemiologisen ympäristön mahdollisia vaikutuksia. Tämä toteutettiin mittaamalla TPX-menetelmällä käytännössä kaikki Suomessa vuosien 1991 ja 2009 välillä eristetyt MRSA-kannat. Tässä laajassa kokoelmassa menetelmä osoittautui sekä herkäksi (97.9 %) että spesifiseksi (94.1 %). Todellisia esikäsittelemättömiä potilasnäytteitä tutkittaessa TPX-menetelmään perustuva MRSA-testi oli positiivinen kaikkien niiden potilaiden kohdalla, joilta MRSA löytyi vertailumenetelmillä. 48 näytettä voitiin mitata kerrallaan. Mediaaniaika todellisen positiivisen tuloksen valmistumiseen oli 3 t 26 min.

Tulosten perusteella voidaan olettaa, että TPX-menetelmään perustuvaa MRSA-testiä voitaisiin tulevaisuudessa hyödyntää suoraan kliinisten potilasnäytteiden tutkimiseen ilman edeltävää puhdasviljelyä. Tällöin testissä negatiivisiksi todettujen, MRSA:lle altistuneiden potilaiden hoitaminen kosketuseristyksessä voitaisiin lopettaa aiempaa nopeammin. Näin päästäisiin mahdollisesti vapauttamaan MRSA:n hallintaan nykyisin sidottuja resursseja. Infektiotilanteissa viive tehokkaan hoidon aloitukseen voisi lyhentyä.

**Avainsanat:** MRSA, TPX, mikrobilääkeherkkyyksien määritys, ison kapasiteetin menetelmät, kaksoisfotonivirittеinen fluoresenssi, vieritestit

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# ABBREVIATIONS

| | |
|---|---|
| ATCC | American Type Culture Collection |
| BHI | Brain-heart infusion broth |
| BORSA | Borderline methicillin-resistant *Staphylococcus aureus* |
| CA | Community-associated |
| CC | Clonal cluster |
| CDC | Center for Disease Control and Prevention |
| CFU | Colony forming unit |
| CLSI | Clinical and Laboratory Standards Institute |
| CoNS | Coagulase-negative *Staphylococcus* |
| DSM | German Collection of Microorganisms and Cell Cultures |
| ELISA | Enzyme-linked immunosorbent assay |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| FDA | United States Food and Drug Administration |
| *F(t)* | Fluorescence-versus-time |
| GISA | Glycopeptide-intermediate *Staphylococcus aureus* |
| GRSA | Glycopeptide-resistant *Staphylococcus aureus* |
| HA | Healthcare-associated |
| HARMONY | European collection of epidemic MRSA strains |
| HICPAC | Hospital Infection Control Practices Advisory Committee |
| IDSA | Infectious Diseases Society of America |
| LA | Livestock-associated |
| LASER | Light amplified by stimulated emission of radiation |
| max(ΔF/Δt) | Maximal fluorescence signal increase rate |
| MDR | Multidrug-resistant |
| *mec* | A gene encoding methicillin resistance in staphylococci |
| MHA | Mueller-Hinton agar |
| MIC | Minimum inhibitory concentration |
| MLST | Multilocus sequence typing |
| MLVA | Multilocus variable-number tandem repeat analysis |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MSA | Mannitol salt agar |
| MSSA | Methicillin-susceptible *Staphylococcus aureus* |
| NPV | Negative predictive value |
| *nuc* | A thermostable nuclease, unique for *Staphylococcus aureus* |
| *OrfX* | A *Staphylococcus aureus*-specific gene sequence |
| PBP2a | An altered penicillin binding protein |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| PFGE | Pulsed-field gel electrophoresis |
| PVL | Panton-Valentine leucocidin |

| | |
|---|---|
| PPV | Positive predictive value |
| ROC | Receiver operating characteristic |
| SIRS | Systemic immune response syndrome |
| SBR | Signal-to-background ratio |
| *SCCmec* | Staphylococcal cassette chromosome *mec* |
| SNRL | Staphylococcus National Reference Laboratory (National Institute for Health and Welfare, Helsinki) |
| *Spa* | A gene encoding *Staphylococcus aureus* protein A |
| SD | Standard deviation |
| ST | Sequence type |
| THL | Terveyden ja hyvinvoinnin laitos (National Institute for Health and Welfare, Finland) |
| THL-NIDR | National Infectious Diseases Register Strain Collection (National Institute for Health and Welfare, Turku) |
| TPX | Two-photon excited fluorescence detection |
| TSB | Tryptic soy broth, a general-purpose growth medium |
| *vanA* | A gene encoding vancomycin resistance |
| VISA | Vancomycin-intermediate *Staphylococcus aureus* |
| hVISA | Heteroresistant VISA |
| VRSA | Vancomycin-resistant *Staphylococcus aureus* |

# LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original papers, referred to in the text by their Roman numerals I-IV.

I. Koskinen J, **Stenholm T**, Vaarno J, Soukka J, Meltola NJ, Soini AE. Development of a rapid assay methodology for antimicrobial susceptibility testing of *Staphylococcus aureus*. Diagn Microb Infect Dis 2008; 62: 306-316.

II. **Stenholm T**, Hakanen AJ, Vaarno J, Pihlasalo S, Terho P, Hänninen PE, Vuopio-Varkila J, Huovinen P, Kotilainen P. Methicillin-resistant *Staphylococcus aureus* screening by online immunometric monitoring of bacterial growth under selective pressure. Antimicrob Agents Chemother 2009; 53: 5088-5094.

III. **Stenholm T**, Hakanen AJ, Salmenlinna S, Pihlasalo S, Härmä H, Hänninen PE, Huovinen P, Vuopio J, Kotilainen P. Evaluation of the TPX MRSA assay for detection of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microb Infect Dis 2011; 30: 1237-1243.

IV. **Stenholm T**, Hakanen AJ, Hakanen E, Härmä H, Rantakokko-Jalava K, Vuopio J, Hänninen PE, Huovinen P, Kotilainen P. High-throughput screening of colonization samples for methicillin-resistant *Staphylococcus aureus*. Submitted, 2012.

# 1. INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is widely accepted as one of the most important multiresistant human pathogens. Contagion with MRSA may at first lead to asymptomatic colonization, but MRSA may later cause a wide variety of infections, ranging from superficial skin infections to rapidly progressing life-threatening disease. The pathogen is capable of causing serious infections in young and healthy individuals, though severe infections commonly develop in hospitalized patients, especially in those with immunocompromising diseases (Vandecasteele, et al., 2009). The risk factors known to predispose a patient for hospital-associated (HA) MRSA comprise operative procedures, treatment in intensive care or trauma units, implanted foreign material, insertion of intravascular catheters or urine catheters, and damaged skin (Coia, et al., 2006). Typical HA-MRSA infections include wound and other skin and soft-tissue infections, bacteremia, pneumonia, and bone and joint infections (Liu, et al., 2011). Severe deep focuses of *S. aureus*, such as endocarditis or spondylodiscitis, often complicate bacteremia (Chang, et al., 2003).

During the 2000s, an increasing proportion of MRSA has been designated as community-associated (CA). Typically, CA-MRSA has caused infection in previously healthy young individuals with no previous contact with healthcare, and no previously identified risk factors for MRSA (DeLeo, et al., 2010). Risk factors for CA-MRSA include e.g., crowding, frequent skin-to-skin contact, and skin diseases. Skin and soft-tissue infections are the most common clinical syndromes caused by CA-MRSA, ranging from impetigo to severe necrotizing fasciitis. CA-MRSA may also cause necrotizing pneumonia (Rubinstein, et al., 2008). Even if CA-MRSA produces a serious disease in some patients, a majority of all cases are not life-threatening.

Fueled by ever increasing consumption of antimicrobial agents MRSA has become endemic in the majority of the developed countries including the USA. More deaths are now associated with MRSA than with susceptible strains of *S. aureus* (MSSA) (Cosgrove, et al., 2003). Patients colonized with MRSA have been reported to be 4 times more likely to develop an invasive infection than those colonized with MSSA (Safdar & Bradley, 2008). In hospitals, the most important route of transmission is direct contact: from MRSA-colonized patients to other patients usually through the hands of hospital personnel, or straight from patient to patient. The main approaches to prevent the spread of MRSA in hospitals include stringent hand hygiene and contact isolation precautions directed at patients colonized with MRSA. Strict infection control policies have been credited with the currently low MRSA prevalence in the Netherlands as well as in the Nordic countries (Tiemersma, et al., 2004). Wide-scale screening of patients with a high risk for MRSA colonization and contact patients of the identified MRSA carriers is considered a cornerstone for implementation of any stringent policy (Bootsma, et al., 2006). Containment of MRSA is considered justified

because, in addition to causing serious illness, MRSA results in significant extra healthcare costs. The measures aimed at controlling nosocomial spread of MRSA are not readily adapted to the control of MRSA in the community.

Rapid high-throughput screening tools are needed to reduce costs associated with maintaining strict MRSA control policies. Traditional screening cultures are still applied in most routine laboratories due to their technical simplicity and low costs per analysis. In low-prevalence settings, MRSA findings are commonly confirmed by the gold standard method: polymerase chain reaction (PCR) focusing on identification of the *mecA* gene. Moreover, complex real-time PCR assays with multiple target sequences, capable of identifying MRSA directly from clinical specimens in a few hours, have been introduced during the 2000s (Huletsky, et al., 2004). Being technically demanding, these sophisticated assays lack sufficient capacity for wide-scale screening purposes. Furthermore, emerging new MRSA clones may not be detectable by such assays resulting in an unacceptably low performance in certain environments (Sissonen, et al., 2009). Quite recently, new MRSA strains have emerged carrying a novel *mecA* gene homologue, *mecC* (*mecA*$_{LGA251}$) (García-Álvarez, et al., 2011). This is of concern since such strains remain undetected by the existing molecular methods.

A 2-photon excited fluorescence (TPX) detection technology was introduced by Hänninen et al. (Hänninen, et al., 2000) for bioaffinity assays. The methodology utilizes polystyrene microspheres as solid-phase reaction carriers for sandwich-type immunocomplex formation (Soini, et al., 2002). Due to the separation-free signal detection, the technique allows online monitoring of bioaffinity reactions (Koskinen, et al., 2006). Our pre-thesis experiments focusing on potential applications of the TPX methodology in the field of bacteriology showed that the unique assay principle allows online monitoring of bacterial growth. A species-specific fluorescence signal could be repeatedly, or even continuously, measured from a turbid and strongly scattering sample without interference with the ongoing reactions. The assay principle also proved suitable for antimicrobial susceptibility testing of bacteria.

This study was commenced to develop a new rapid and cost-effective method based on the TPX methodology for the screening of MRSA directly from clinical colonization samples. The early phases of this work were aimed at verifying that the TPX MRSA assay principle is adequate for the detection of MRSA, and that all technical demands associated with the TPX procedure can be met. Also, efforts were made to improve and optimize the assay performance by adoption of the best possible assay reagents, hardware, and test protocols. Later, the TPX assay tolerance to wide biological variation was determined by evaluating a large collection of Finnish epidemic MRSA isolates. Finally, the applicability of the TPX MRSA assay to high-throughput screening of MRSA directly from colonization samples was assessed.

# 2. REVIEW OF THE LITERATURE

## 2.1. *Staphylococcus aureus*

### 2.1.1. The history of *S. aureus*

Staphylococci were first observed and cultured by Robert Koch (1843-1910) and Louis Pasteur (1822-1895) but the first detailed studies were published by a Scottish surgeon Sir Alexander Ogston (1844-1929) a few years later (Ogston, 1880; Ogston, 1882). He viewed clusters of round organisms under the microscope and demonstrated their causative role in abscess formation (Ogston, 1880). The Latin name *Staphylococcus* was given by Sir Ogston to this family of pus forming bacteria based on their appearance under the microscope.

Shortly after Sir Ogston's discovery another surgeon, a German Anton J. Rosenbach (1842-1923), was able to isolate and grow *Staphylococcus aureus* (Rosenbach, 1884). He named the species after the yellowish color of the colony (from Latin: *aurum* "gold"). The tube coagulase test for differentiation of *S. aureus* from the less virulent staphylococci was introduced in the 1930s (Chapman, et al., 1934).

In 1928, the bacteriologist Sir Alexander Fleming (1881-1955) observed that *S. aureus* could not grow in the presence of the mold *Penicillium notatum* (Fleming, 1929). However, it was not until 10 years later that penicillin was purified and large enough quantities could be produced to begin treatment trials. The first animal experiments were conducted in Oxford in 1939 and the results were published by the *Lancet* next year (Chain, et al., 1940). The first human trial soon followed (Abraham, et al., 1941). By the fall of 1943 enough penicillin was available to satisfy the huge demand of the Allied war efforts (Riley, 1972). Before the introduction of penicillin mortality in *S. aureus* bacteremia had exceeded 80% (Skinner & Keefer, 1941).

### 2.1.2. Classification of staphylococci and identification of *S. aureus*

The laboratory diagnosis of staphylococci is based on direct microscopy of a gram stain, culture and various biochemical tests. Under the microscope staphylococci are gram-positive spherical bacterial cells that grow in 3 dimensions forming grape-like clusters. Direct microscopy by itself cannot adequately differentiate between e.g., different species of staphylococci, enterococci and streptococci. Fluorescence *in situ* hybridization (FISH) with probes targeting *S. aureus* ribonucleic acids (RNA) have been utilized to allow reliable identification by direct microscopy (Oliveira, et al., 2003).

All species of the genus *Staphylococcus* are facultative anaerobes, i.e. capable of growing in both aerobic and anaerobic environments. They can grow in temperatures ranging from 15°C to 45°C and at high NaCl concentrations up to 15%. A catalase test can be used to differentiate *Staphylococcus* colonies from other gram-positive bacteria: catalase-positive species such as staphylococci form bubbles of oxygen gas when a colony comes into contact with hydrogen peroxide ($2\ H_2O_2 \Rightarrow 2\ H_2O + O_2$). Catalase-negative species such as streptococci and enterococci do not provoke a detectable reaction.

On a blood agar plate *S. aureus* typically forms large yellow colonies accompanied by a surrounding hemolytic zone. Other staphylococci form small white colonies without the surrounding hemolysis. Mannitol Salt Agar (MSA) is commonly used for pure culture of staphylococci. It contains mannitol, a pH-indicator called phenol red, and salt (NaCl 7.5-10%). *S. aureus* is able to ferment mannitol, which results in an acidic by-product and a consequent change in the indicator color to yellow. Most clinically significant coagulase-negative staphylococci (CoNS), with the exception of *S. saprophyticus*, are unable to use mannitol and the color of the MSA plate remains red around such colonies. A summary of classical laboratory tests for differentiation of staphylococci is presented in Table 1.

**Table 1.** Classical laboratory tests for differentiation of clinically important staphylococci*

| Test | *S. aureus*[a] | *S. epidermidis*[b] | *S. saphrophyticus*[c] |
|---|---|---|---|
| Coagulase test | + | - | - |
| Catalase test | + | + | + |
| Mannitol fermentation | + | - | + (-) |
| DNAse test[d] | + | - | - |
| Resistance to novobiocin[e] | - | - | + |
| Hemolysis | + | - (+) | - |

An uncommon result in brackets

[a] *S. aureus*, *Staphylococcus aureus*

[b] *S. epidermidis*, *Staphylococcus epidermidis*

[c] *S. saphrophyticus, Staphylococcus saphrophyticus*

[d] DNAse test, colonies that produce DNA-cleaving enzymes form a clear zone on DNAse agar

[e] Novobiocin, an aminocoumarin antibiotic

**According to Vuopio-Varkila, et al,. 2010*

A generally accepted criterion for the identification of *S. aureus* is a positive coagulase test. Coagulase is an enzyme that converts fibrinogen into fibrin, i.e. causes blood clotting. *S. aureus* produces both free coagulase and coagulase bound on cell surfaces while CoNS do not express the protein. The tube coagulase test is considered the gold

standard for distinguishing a coagulase-positive species from the generally less virulent CoNS: rabbit plasma is inoculated with a staphylococcal colony and incubated at +37ºC for up to 18 h. If a clot is formed, the test is positive for the presence of a coagulase-positive species. To date, 6 species of coagulase-positive staphylococci have been identified in addition to *S. aureus*. These include *S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini* and *S. pseudointermedius* (Sasaki, et al., 2010). Coagulase-positive species other than *S. aureus* are predominantly animal pathogens (Igimi, et al., 1990; Sasaki, et al., 2010). In clinical practice *S. epidermidis*, *S. haemolyticus* and *S. hominis* are the most frequently encountered CoNS species with more than 50% of clinical isolates belonging to the species *S. epidermidis* (Piette & Verschraegen, 2009). The current number of identified CoNS species is 36 (Coton, et al., 2010). CoNS rarely cause disease in otherwise healthy individuals (Rogers, et al., 2009), and they were considered mere contaminants until the late 1950s.

The first generation of latex agglutination tests for rapid identification of *S. aureus* colonies detected bound coagulase and protein A on bacterial cell surfaces (Essers & Radebold, 1980; Myrick & Ellner, 1982; Rossney, et al., 1990). A new generation of highly sensitive commercial slide agglutination tests for identification of *S. aureus* was based on monoclonal antibodies against multiple antigens, including the capsular polysaccharides (Fournier, et al., 1993). These tests still suffer from false-positive results due to some CoNS strains known to share the same antigens with *S. aureus* (Blake & Metcalfe, 2001).

Simple PCR assays for reliable identification of *S. aureus* colonies have been around for 2 decades (Brakstad, et al., 1992). The *nuc* gene, which encodes a unique thermostable nuclease, is specific for the pathogen. Although expensive and laborious, sequence analyses of several different target genes are considered the most reliable techniques for identification of the genus *Staphylococcus* at the species level (Kwok & Chow, 2003). Multiplex-PCR methods more suitable for routine laboratory use have been developed recently (Sasaki, et al., 2010; Hirotaki, et al., 2011).

### 2.1.3. *S. aureus* carriage

The anterior nares are the most frequent site of asymptomatic *S. aureus* carriage in humans. About 20% of the population are persistent carriers, while approximately 30% carry the organism only intermittently (Kluytmans, et al., 1997; Wertheim, et al., 2005). Children have higher persistent carriage rates than adults (Wertheim et al., 2005). In patients with skin lesions or atopic dermatitis, a much higher rate of nasal carriage (70%) is found (Hoeger, et al., 1992). Other sites of colonization include the perineum, vagina, throat, axillae/groin, and intertriginous skin folds (Ridley, 1959; Solberg, 1965; Coello, et al., 1994). The carriage of *S. aureus* is usually highest in hospitalized patients. The organism is also found on clothing, bed linen, and other

foamites of human environments. *S. aureus* can be spread to other individuals in hospitals and in the community from healthy carriers among patients and personnel as well as from infected persons. The most important route of transmission is direct contact via the hands of hospital staff. It can also spread by contamination from the environment, or by aerial dissemination especially from skin and upper respiratory tract (Solberg, 2000).

### 2.1.4. Virulence mechanisms

*S. aureus* is equipped with a wide arsenal of virulence mechanisms (Table 2). These enable abscess formation, evasion of host immune responses at many levels, and induction of the sepsis syndrome (Raygada & Levine, 2009). For example protein A binds immunoglobulins on *S. aureus* surfaces and is thought to act as an immunologic disguise (Forsgren & Nordstrom, 1974). Nearly all strains secrete a variety of proteins to the surrounding media that convert host tissues to nutrients for the invading bacterium (Dinges, et al., 2000). Some strains also produce toxins, which have systemic effects. Fever, hypotension, rashes, and emesis are all clinical manifestations of circulating toxins.

**Table 2.** *Staphylococcus aureus* virulence mechanisms

| Virulence factor | Description | Role | Clinical consequences |
|---|---|---|---|
| clumping factors | surface proteins | attachment to host tissues | endocarditis, foreign body infections |
| fibrinogen binding protein<br>collagen binding protein | MSCRAMMas | | septic arthritis |
| proteases<br>lipases<br>nucleases<br>hyaluronidases<br>elastase | invasines | penetration into host tissues | invasion and destruction |
| haemolysin | cytotoxins | lysis of red blood cells | |
| PVL[b] | | destruction of leucocytes | necrotizing pneumonia |
| protein A | | evasion of opsonization | metastatic infections |
| capsular polysaccharides | | biofilm production | persistent infections, abscess formation |
| catalase<br>staphyloxanthin | antioxidants | reduction of reactive oxygen | increased survivability |
| TSST[c] | exotoxins | releases cytokines | toxic shock syndrome |
| enterotoxins | | gastrointestinal toxins | food poisoning |
| exfoliative toxins | | cleaves epidermal structures | scalded skin syndrome |
| penicillinase | resistance determinants | cleaves penicillin | penicillin resistance |
| PBP2a[d] | | altered antibiotic binding site | methicillin resistance |
| *VanA*[e] | | altered antibiotic binding site | vancomycin resistance |
| efflux pumps | | exocytosis of drug molecules | aminoglycoside resistance |
| modifying enzymes | | inactivation of drug molecules | aminoglycoside resistance |

[a]MSCRAMM, microbial surface components recognizing adhesive matrix molecules
[b]PVL, Panton-Valentine leucocidin
[c]TSST, toxic shock syndrome toxin
[d]PBP2a, an altered penicillin binding protein encoded by the *mecA* gene
[e]*VanA*, a gene encoding vancomycin resistance

*Modified from Raygada & Levine, 2009*

Similar symptoms may develop without bacteremia in the toxic shock syndrome, where a patient is intoxicated by *S. aureus* metabolic by-products absorbed from a body cavity (Schlievert, et al., 2004). Food poisoning may also be caused by staphylococcal enterotoxins (Marrack & Kappler, 1990). Panton-Valentine leucocidin (PVL) is a plausible virulence factor associated with destruction of leucocytes. The capacity to excrete PVL is characteristic of *S. aureus* strains which are resistant to methicillin and circulate outside hospitals (DeLeo, et al., 2010), although not totally restricted to such strains (Tinelli, et al., 2009).

### 2.1.5. Clinical features of infections

*S. aureus* infections form a permanent threat to humans, being associated with high morbidity and mortality (Sheagren, 1984). It is the leading cause of lethal bacteremic infections and second only to CoNS as a cause of nosocomial bacteremia (Emori & Gaines, 1993). The pathogen has also been credited with being the most common cause of all hospital-acquired infections (Archer, 1998). Diabetes mellitus, vascular access sites, intravenous drug abuse, and immunodeficiency have been identified as predisposing factors for *S. aureus* infections, although perfectly healthy individuals can be infected as well (Lowy, 1998). ). In the community, *S. aureus* is a causative agent of e.g., superficial skin infections (impetigo, folliculitis, furunculosis), soft-tissue infections, conjunctivitis, and sometimes sepsis, septic arthritis or infective endocarditis. In hospital, wound infections and other surgical site infections, bone and joint infections, bacteremia, and pneumonia are common presentations of *S. aureus* infections.

The pathogenesis of *S. aureus* infections is divided into 5 stages (Archer, 1998): colonization, local infection, systemic dissemination, metastatic infection and, finally, toxic shock. Healthy skin presents an effective barrier, but through small abrasions a local skin infection may develop. Unless limited by the host immune defences, the infection advances into a local abscess and eventually gains access to the blood stream. Through blood the organism can spread to any distant organ causing endocarditis, osteomyelitis, deep abscesses, etc. Septic shock may ensue as a result of systemic effects of circulating toxins. Definitions for the associated systemic immune response syndrome (SIRS), sepsis, and septic shock are found in Table 3 (ACCP, 1992). These criteria have been widely considered to be too nonspecific while the later SIRS definitions have become increasingly complicated (Levy, et al., 2003). Despite the best efforts of modern medicine, a third of patients with a disseminated *S. aureus* infection still die within a month (Fowler, et al., 1998). In a Finnish prospective multicenter trial involving 381 patients with *S. aureus* bacteremia between 2000-2002, case fatality rate was 14% at 28 days and 19% at 3 months (Ruotsalainen, et al., 2006). Deep infection was found in 84% of the patients within 1 week following the commencement of effective antimicrobial treatment. All patients received at least 14 days of intravenous antimicrobial treatment.

**Table 3.** Definitions of SIRS[a], sepsis, severe sepsis, and septic shock*

| | |
|---|---|
| SIRS | Body temperature >38°C or < 36°C<br>Heart rate > 90/min<br>Respiratory rate > 20/min or pCO2 < 4.3 kPa<br>White blood cell count > $12x10^9$ or < $4 x10^9$/L<br>or >10% of total leucocytes immature |
| Sepsis | Clinical signs of SIRS associated with confirmed infection |
| Severe sepsis | Presence of sepsis with at least 1 sign of hypoperfusion or organ dysfunction |
| Septic shock | Mean arterial pressure below 60 mmHg (or 80 mmHg for the previously hypertensive)<br>or inotrope infusion required to maintain systolic blood pressure above the same level |

[a]SIRS, systemic inflammatory response syndrome

**According to the Society of Critical Care Medicine and the American College of Chest Physicians (ACCP), 1992*

### 2.1.6. Antimicrobial resistance and treatment options for infections

The first report of *S. aureus* resistance to penicillin through the acquisition of genes producing beta-lactamase (penicillinase) came out even before the mass production (Rammelcamp & Maxon, 1942). The emergence of penicillin resistance led to the introduction of the semisynthetic penicillinase-stable antistaphylococcal penicillins. The first of them, methicillin, was introduced for the treatment of *S. aureus* infections in 1961, being followed by other derivatives including oxacillin, cloxacillin and dicloxacillin.

By the late 1960s more than 80% of *S. aureus* isolates had developed resistance to penicillin (Lowy, 2003). Thus, penicillin was no longer an option for the antimicrobial treatment of *S. aureus* infections. The cornerstone of the therapy of severe diseases, such as MSSA bacteremia, infective endocarditis, and deep metastatic foci, is intravenous oxacillin or cloxacillin (Paul, et al., 2011). Milder MSSA infections are commonly treated with oral cephalosporins, clindamycin, oral antistaphylococcal penicillins, or sometimes with fluoroquinolones. Superficial MSSA skin infections may be cured with only locally applied fusidic acid.

## 2.2. Methicillin-resistant *S. aureus* (MRSA)

### 2.2.1. Emergence of methicillin resistance and resistance mechanisms

The first reports of methicillin resistance in *S. aureus* appeared soon after the introduction of penicillinase-stable penicillins (Jevons, 1961). Methicillin resistance in staphylococci is caused by the *mecA* gene which encodes an altered penicillin-binding

protein 2a (PBP2a) with a low affinity for beta-lactam antibiotics such as penicillin, antistaphylococcal penicillins including methicillin, and cephalosporins (Chambers, 1997). MRSA are generated when MSSA acquire the *mecA* gene. Recently, a new *mecA* gene homologue, $mecA_{LGA251}$ in reference to the *S. aureus* LGA251 isolates from which it was described, has also been found to convey methicillin resistance. (García-Álvarez, et al., 2011). It was recently renamed *mecC* (Laurent, et al., 2012; Petersen, et al., 2012; Ito, et al., 2012).

The staphylococcal cassette chromosome *mec* (*SCCmec*) is a family of large mobile genetic elements that include the *mecA* gene (Ito, et al., 2001). The *mecA* MRSA strains are thought to have emerged by means of horizontally transferred *SCCmec* from CoNS (Forbes & Schaberg, 1983; Wielders, et al., 2002). A majority of nosocomial CoNS, the foremost of them *S. epidermidis*, has already for years been resistant to methicillin based on carriage of the *mecA* gene (Archer & Climo, 1994). Earlier studies revealed substantial homology between *S. aureus mecA* and the *mecA* genes found in the coagulase-negative *S. sciuri* group, which is mainly isolated from animals and foodstuffs (Couto, et al., 1996). More recent studies have demonstrated that of the 3 *mecA*-positive *S. sciuri* species groups (*S. sciuri*, *S. vitilinus*, and *S. fleurettii*), the *mecA* gene of *S. fleurettii* shows *in vitro* methicillin resistance and has the highest homology (> 99% nucleotide identity) with the *mecA* gene of the present-day MRSA strain N315 (Tsubakishita, et al., 2010). This clearly suggests that *S. fleurettii* rather than *S. sciuri* might have been the source of *mecA* (Moellering, 2012). On the other hand, a transfer of DNA containing the *mecA* code from *S. epidermidis* to *S. aureus* has been witnessed *in vivo* (Wielders, et al., 2001). A vancomycin-resistant *S. aureus* (VRSA) strain equipped with a comparable mobile genetic element for effective transfer of the resistance mechanism has been described at the beginning of the 2000s (Centers for Disease Control and Prevention, CDC, 2002).

A characteristic element of methicillin resistance is its heterogeneous nature, as the level of resistance varies in different culture conditions and antimicrobial environments (Chambers, 1997). Some MRSA strains termed as heterogeneous are composed of 2 sub-populations: a majority of phenotypically susceptible cells and a small minority (typically 0.01%) of highly resistant cells (Hartman & Tomasz, 1986; Tomasz, et al., 1991). Most clinical isolates show the heterogeneous pattern under standard growth conditions. Heterogeneous strains can also show a homogeneous resistance pattern under particular culture conditions, e.g., in hypertonic culture medium supplemented with NaCl. Under antibiotic pressure the highly resistant subpopulation may be selected resulting in a homogeneous population of highly resistant strains, and a potential treatment failure.

Borderline (or low-level) methicillin-resistant strains are characterized by methicillin MICs at or just above the susceptibility breakpoint (e.g., oxacillin MICs of 4 to 8

mg/L). Such strains may be divided into 2 groups based on whether the *mecA* gene is present. Borderline strains with *mecA* are extremely heterogeneous methicillin-resistant strains that produce PBP2a (Chambers, 1997). The resistant subpopulation may be very small, but grows under antibiotic pressure. Borderline methicillin resistance may also be due to mechanisms other than PBP2a production (Chambers, 1997). Strains with non-*mecA*-mediated borderline resistance are known to produce excessive amounts of beta-lactamase which hydrolyze oxacillin (McDougal & Thornsberry, 1986), and they may become entirely oxacillin-susceptible in the presence of beta-lactamase inhibitors. The non-*mecA* strains are characterized by the absence of highly resistant clones (Chambers, 1997). The phenotype may also be caused by other mechanisms, such as production of plasmid-mediated methicillinase or different modifications in the PBP genes (Montanari, et al., 1996; Nadarajah, et al., 2006). There is no clinical data proposing that resistance exhibited by these borderline-resistant *mecA*-negative *S. aureus* strains has been of any clinical significance for the treatment of infections (Chambers, et al., 1989; Chambers, 1997). Borderline oxacillin-resistant *S. aureus* has been infrequently termed as BORSA (Jorgensen, 1991; Liu & Lewis, 1992). Some authors still restrict the use of this abbreviation for *mecA*-negative *S. aureus* strains with oxacillin MICs of 2 to 8 mg/L (Louie, et al., 2000).

### 2.2.2. Epidemiology, typing methods, and clinical infections

Since their description in 1961, MRSA clones have spread around the world reaching an endemic status in most of the developed countries. It is not known whether this is due to differentiation from only 1 specific clone or introduction of *SCCmecA* into numerous clones (Moellering, 2012). Until lately, MRSA clones have mainly been of hospital or health care-associated (HA) origin. MRSA infections in persons with no previous history with healthcare contact were first reported in the early 1990s from Australia, in the late 1990s from the USA, and in the early 2000s from Europe (Udo, et al., 1993; Chambers & DeLeo, 2009; Otter and French, 2010). Due to the absence of consensus on the definition and nomenclature of these newly recovered MRSA types, different authors have used different classifications. Initially, the strains were termed community-acquired MRSA, but presently, the most common term used is community-associated (CA) MRSA, to manifest the ambiguity as to whether the strain has been acquired in the community or in hospital. Indeed, HA-MRSA and CA-MRSA strains cannot be reliably differentiated based on epidemiological data alone, indicating a need for a genotypic definition. In recent years, MRSA has also been isolated from livestock (livestock-associated MRSA, LA-MRSA) (Lee, 2003; Voss, et al., 2005; van Cleef, et al., 2011). Today, pandemic MRSA includes dissemination of HA-MRSA clones from the 1960s, CA-MRSA clones from the 1990s, and LA-MRSA clones from the 2000s (Chambers & DeLeo, 2009; DeLeo & Chambers, 2009; van Cleef, et al., 2011, Stefani, et al., 2012).

#### 2.2.2.1. Tools for epidemiological typing of MRSA

Multiple laboratory tools for monitoring MRSA epidemiology have been developed (Struelens, et al., 2009). Up to now, no single typing method has provided sufficient information for discrimination required e.g., in MRSA outbreak investigations and surveillance programs. The characteristics of the currently most common typing methods, with their strengths and weaknesses, are listed in Table 4.

1) Pulsed-field gel electrophoresis (PFGE) of fragmented *S. aureus* DNA is a widely used method in local MRSA epidemics. The major disadvantage of PFGE lies in subjective interpretation of fragmentation patterns, and comparing the results from different laboratories has proven difficult. PFGE is also technically difficult (Struelens, et al., 2009).

2) *Spa* typing utilizes polymorphism in the variable region of the gene encoding *Staphylococcus aureus* protein A (*Spa*). The technique has a high-throughput capacity and a standardized nomenclature. The discriminatory power of *Spa* typing in regions with a small number of epidemic clones may be insufficient (Struelens, et al., 2009).

3) Multilocus sequence typing (MLST) is based on sequencing of 7 housekeeping genes in the *S. aureus* genome. It is highly unlikely that 2 unrelated isolates would have identical allelic profiles at all of the 7 loci by chance (Enright, et al., 2000). A sequence type (ST) number is assigned to those isolates that are identical by MLST and closely related STs are grouped into clonal clusters (CC). Unlike in PFGE, variations accumulate relatively slowly within the housekeeping genes sequenced in MLST. Isolates with the same MLST profile may be descended from a common ancestor that existed years ago (Enright, et al., 2000). The main weaknesses of MLST are a low-throughput and high cost per analysis.

4) Sequencing of the *SCCmec* is also used in epidemiological typing (Deurenberg, et al., 2005). Currently 5 main types are identified (I-V). There is a need for harmonizing several *SCCmec* typing schemes although the nomenclature is defined. A low-throughput capacity is also a difficulty.

5) Multilocus variable-number tandem repeat analysis (MLVA) is a high-throughput method increasingly utilized instead of PFGE (Struelens, et al., 2009). There is no standardized nomenclature and multiple schemes are currently used. MLVA appears to have a higher discriminatory power than PFGE or *Spa* sequencing (Stefani, et al., 2012).

**Table 4.** Tools for epidemiological typing of methicillin-resistant *Staphylococcus aureus*

| Technique | Principle | Strengths | Weaknesses |
|---|---|---|---|
| PFGE[a] | Comparison of restriction patterns of the whole microbial genome | High discriminatory power | Slow, technically difficult, subjective, multiple nomenclatures |
| MLST[b] | Sequencing of 7 conserved "housekeeping" genes | Well reproducible, standard nomenclature | Limited discriminatory power low throughput, high cost |
| SLST[c] *Spa*-typing[d] | Polymorphism of the hypervariable region of the gene encoding protein A | Interlaboratory portability, rapid, high throughput | Moderate discriminatory power |
| SCC-*mec*[e] typing | Typing of multiple loci of the SCC*mec* chromosome | Defined nomenclature, high discriminatory power | Low throughput capacity, need for harmonized typing schemes |
| MLVA[f] | Polymorphisms in chromosomal variable number tandem DNA repeats | High throughput, rapid, high discriminatory power | No standard nomenclature, no validated interpretation |

[a]PFGE, pulsed field gel electrophoresis
[b]MLST, multilocus sequence typing
[c]SLST, single locus sequence typing
[d]*Spa*, *Staphylococcus aureus* protein A
[e]SCC*mec*, staphylococcal cassette choromosome carrying the methicillin-resistance associated *mecA* gene
[f]MLVA, multilocus variable-number tandem repeat analysis

*Modified from Struelens, et al., 2009*

### 2.2.2.2. Risk factors, modes of transmission, and clinical infections

#### *2.2.2.2.1. HA-MRSA*

Factors predisposing a patient to colonization with HA-MRSA correspond to those rendering a patient at risk for nosocomial colonization with MSSA. Patients undergoing extensive operative procedures or who are treated in burns, intensive care, or trauma units are prone to become colonized with HA-MRSA (Coia, et al., 2006). Similarly patients with implanted foreign material and those with different immunocompromising diseases have an increased risk for MRSA. Damaged skin (e.g., wounds, eczema) and insertion of intravascular catheters further increase the risk for colonization (Coia, et al., 2006). The anterior nares may be persistently or intermittently colonized with MRSA whereas carriage at other normal body sites is generally less frequent and persistent (Sanford, et al., 1994). Colonization of the throat may be a marker of persistent carriage (Coia, et al., 2006).

Transmission of HA-MRSA occurs most commonly through the hands of hospital workers from MRSA-colonized or infected patients to other patients. As well, direct patient to patient transmission of HA-MRSA is possible. Patients can also contract MRSA from contaminated inanimate environment or, sometimes, through aerial dissemination from heavy MRSA disperses (Sohlberg, 2000).

HA-MRSA strains are almost always acquired during healthcare contact, but the onset of infection may be in hospital or in the community. The variety and clinical presentation of infections caused by HA-MRSA correspond to those caused by MSSA

during hospital stay. These infections include e.g., wound and other skin and soft-tissue infections, bacteremia and endocarditis, pneumonia, bone and joint infections, prosthetic device-associated infections, and central nervous system infections (Liu, et al., 2011). Serious MRSA disease is associated with high morbidity, and mortality rates between 30% and 37% have been reported for patients with MRSA endocarditis (Miro, et al., 2005; Fowler et al., 2005). The high mortality may at least partly be due to a delay in the commencement of effective antimicrobial therapy.

#### *2.2.2.2.2. CA-MRSA*

CA-MRSA by definition affects patients with no prior contact with healthcare settings. Initially, CA-MRSA were considered to be MRSA strains which cause infection in previously healthy young individuals with no identified risk factors for acquisition of MRSA. An epidemiological definition of CA-MRSA proposed by the CDC is as follows: MRSA must be identified in the outpatient setting or less than 48 h after hospital admission in an individual with no medical history of MRSA infection or colonization, admission to a healthcare facility, dialysis, surgery, or insertion of indwelling devices in the past year (Millar, et al., 2007). Early data also implied that these strains were susceptible to most non-beta-lactam antimicrobial agents and carried genes encoding PVL (Chambers & DeLeo, 2009).

The absence of conventional risk factors for MRSA in patients colonized with CA-MRSA suggests that some of these strains may spread more easily than customary HA-MRSA strains (DeLeo, et al., 2010). Like all *S. aureus* strains, CA-MRSA is transmitted by direct contact with infected or colonized individuals, or through a MRSA-contaminated environment (DeLeo, et al., 2010). CA-MRSA can be acquired through activities in which direct body contact is common. The CDC has proposed 5 factors (or 5 Cs) associated with CA-MRSA transmission, i.e. 1) Crowding, 2) frequent skin-to-skin Contact, 3) Compromised skin integrity, 4) Contaminated items and surfaces, and 5) lack of Cleanliness. These factors are common in populations with increased numbers of infections or colonization caused by CA-MRSA (DeLeo, et al., 2010). Wrestlers, football players, prison inmates, soldiers, children in day-care centers, etc. have been identified as high risk groups for CA-MRSA (Liu, et al., 2008).

The clinical picture caused by CA-MRSA differs from that caused by HA-MRSA in many respects. In contrast to other *S. aureus* strains, a majority of CA-MRSA in the USA carry genes encoding the PVL (Diep, et al., 2004), although PVL is not present in all CA-MRSA strains. PVL is known to target and injure the membranes of polymorphonuclear leucocytes. The CA-MRSA strains producing PVL are associated with tissue necrosis and abscess formation (Lina, et al., 1999) and yet, animal studies on the clinical significance of PVL as a virulence factor have yielded conflicting results (Lowy, 2011).

Skin and soft-tissue infections prevail among the clinical syndromes caused by CA-MRSA, ranging from impetigo to severe necrotizing fasciitis (Miller, et al., 2005). The most common lesions are abscesses and cellulitis (Miller, et al., 2007). CA-MRSA is also a frightening cause of pneumonia. CA-MRSA pneumonia is linked with an influenza-like illness, often affects young and previously healthy individuals, and often takes a violent course leading to an abundance of complications and high mortality rates (Rubinstein, et al., 2008). Although debatable, PVL has been considered a virulence factor in association with severe necrotizing pneumonia. Based on the capacity of CA-MRSA to cause infections in otherwise healthy individuals and to lead to unusually severe disease it has been speculated that CA-MRSA strains may have a greater virulence and power to evade host defenses than do customary HA-MRSA strains (DeLeo, et al., 2010). This assumption is supported by the increased virulence of epidemic CA-MRSA as compared with HA-MRSA in models of animal infections (Li, et al., 2009). Even if CA-MRSA produces a formidable disease in some of the patients, a majority of all cases with CA-MRSA are not life-threatening.

As compared to HA-MRSA, many of the CA-MRSA strains have less often been multiresistant (MDR), i.e. resistant to more than 3 different antimicrobial groups (Chambers & DeLeo, 2009; Otter & French, 2010), but the situation is dynamic and may rapidly change.

It is important to differentiate between CA-MRSA and HA-MRSA in order to provide optimal clinical treatment and infection control measures, as well as to reliably monitor the epidemiological MRSA situation worldwide. In recent years, knowledge on how to make a distinction between CA-MRSA and HA-MRSA has increased. For classification, epidemiological data is of value, but may yield confounding results when used alone (Otter & French, 2012). For example, an MRSA infection manifesting in a community setting may be caused by an MRSA strain acquired by some earlier hospital stay, but become falsely identified as CA-MRSA based on epidemiological information. Illustratively, most bacteremias diagnosed at hospital admission, and therefore classified as CA-MRSA infections, have been caused by HA-MRSA from a previous healthcare contact (Miller, et al., 2008). On the other hand, MRSA infections developing during hospital stay may be caused by MRSA acquired in the community. Epidemiological classification was more useful in the past than nowadays. Although HA-MRSA is still uncommonly transmitted outside hospitals, CA-MRSA clones have begun to spread in hospitals. Carriage of PVL and susceptibility to non-beta-lactam antimicrobial agents may be useful markers of CA-MRSA, but cannot be used for designation (Otter & French, 2012).

CA-MRSA strains are usually common community types of MSSA which have acquired *mecA de novo* (Enright, et al., 2002). Therefore, it has been suggested by Otter and French (Otter & French, 2012) that currently the best way to define CA-

MRSA strains may be to combine a genotyping method (e.g., MLST, *spa*, or PFGE) with SCC*mec* analysis to conclude the likely origin of an MRSA strain. It is not so complicated to obtain a genotypic definition in the USA where e.g., USA300 is prominent among CA-MRSA, while developing a genotypic classification is much more difficult in Europe or Australia where CA-MRSA are genotypically heterogeneic (Otter & French, 2012). The most common types of CA-MRSA and HA-MRSA wordwide are presented below.

#### *2.2.2.2.3. LA-MRSA*

In 2004, an MRSA strain ST398 was recognized from pigs and healthy pig farmers in the Netherlands, and the clonal spread between man and pigs was clearly demonstrated by epidemiological typing methods (Huijsdens, et al., 2006). Consequently, occupational exposure to animals was recognized as a new factor predisposing to MRSA colonization (van Loo, et al., 2007; Lewis, et al., 2008). LA-MRSA has been isolated mainly from pigs and to a lesser extent from cattle and poultry (Verhegghe, 2012).

LA-MRSA was connected with human illness in 2003, when an MRSA clone present in a reservoir of pigs and cattle was isolated from a human (Huijsdens, et al., 2006). Although LA-MRSA is an uncommon causative agent of infections, it is necessary to be on the alert for emerging animal strains of MRSA and their potential association with human infections. MRSA ST398, habitually associated with colonization and infections of pigs and other farm animals, has been isolated from human samples in several countries (van Cleef, et al., 2011). It is of note that the proportion of MRSA ST398 isolated from blood has been significantly lower than other MRSA, suggesting less serious disease. In recent years, however, MRSA ST398 has reportedly been a cause of severe infections among pig farmers (Lowy, 2011).

### 2.2.2.3. Prevalence and types of MRSA in different countries

According to a recent review, the prevalence of HA-MRSA around the world is as follows (Stefani, et al., 2012): the highest rates (> 50%) of HA-MRSA as a proportion of all *S. aureus* infections are reported from North and South America, Asia and Malta. Intermediate rates of 25-50% are reported from China, Australia, Africa, Portugal, Greece, Italy, Hungary, Spain, Ireland, Romania, and the United Kingdom. The Netherlands and the northern European countries stand out with their low rates of < 5% while the prevalence in the rest of Europe is currently 5-25%. The proportion of HA-MRSA infections has significantly declined in recent years in Austria, France, Ireland, and the UK while it has remained stable in other European countries. The most frequently reported MLST CCs from all continents are CC5, CC8, CC22, CC30, and CC45 (Campanile, et al., 2010). CC5 and CC8 are the most prevalent worldwide. In

general, *SCCmec* types I-III are associated with HA-MRSA. Type I encodes beta-lactam resistance while types II and III determine multiresistance.

From the early 2000s up to now, an increasing proportion of MRSA has been recognized to be community-associated (Chambers, 2001; DeLeo, et al., 2010; Stefani, et al., 2012). The number of cases of CA-MRSA has rapidly escalated especially in the USA: in the San Francisco area in 2004-2005, the incidence of CA-MRSA was already 10-fold higher than the incidence of HA-MRSA (Liu, et al., 2008). A vast majority of the CA-MRSA isolates are of a single strain, the USA300 (CC8-ST8-*SCCmec*IV). The USA300 strain is currently considered indicative of an infection of community origin (Tenover & Goering, 2009). CA-MRSA has remained uncommon in Europe with a different predominant clone: CC80-ST80-*SCCmec*IV. In general, *SCCmec* types IV-V are associated with CA-MRSA. Neither of the 2 *SCCmec* types determines multiresistance, but it can be acquired by other genetic mechanisms.

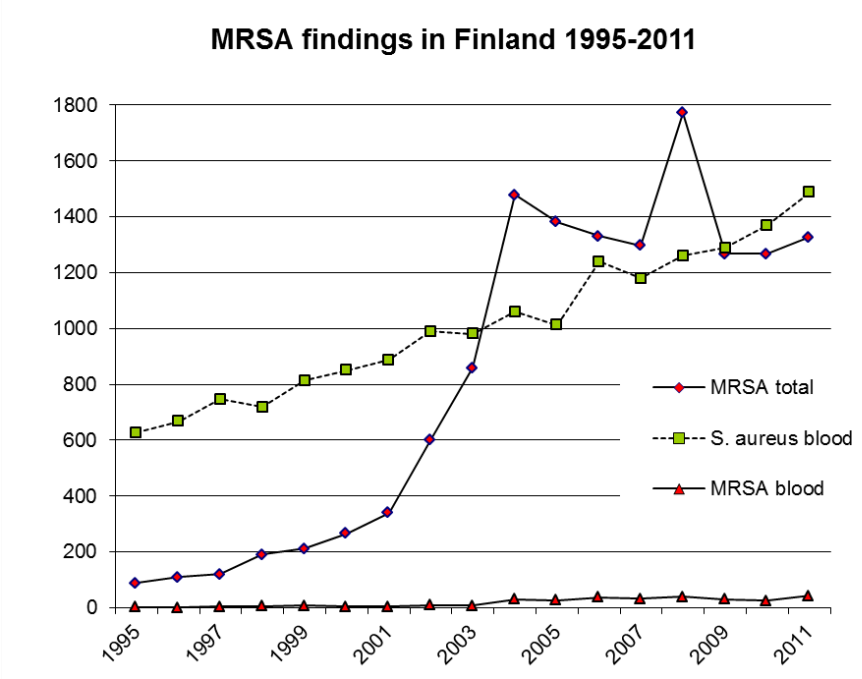


FIGURE 1. Methicillin resistant *S. aureus* (MRSA) findings in Finland 1995-2011 according to National Institute for Health and Welfare (THL), Finland (http://www.ktl.fi/portal/suomi/terveyden_ammattilaisille/infektiotaudit/tartuntataudit_suomessa_-julkaisut/). MRSA total; all MRSA findings reported to THL including asymptomatic colonization. MRSA blood; MRSA blood culture findings. *S. aureus* blood: All *S. aureus* blood findings including sensitive isolates.

The MRSA findings in Finland during 1995-2011 are described in Figure 1. According to a nation-wide register of infectious diseases kept by the National Institute for Health and Welfare (THL), Finland, the total number of MRSA findings reached 1267, 1267, and 1327 in 2009, 2010 and 2011, respectively (www.thl.fi). In recent years, the proportion of MRSA from all *S. aureus* isolates from blood and pus cultures has stabilized at the level of 2-3% (www.finres.fi) being, thus, somewhat higher than e.g., in Sweden and Norway. Based on the THL register, the proportions of MRSA from all *S. aureus* blood isolates were 2.3% (30/1288), 1.9% (26/2370), and 2.8% (42/1487) in 2009, 2010 and 2011, respectively (www.thl.fi). Regional differences are noticeable (Laine, et al., 2012), and in most hospitals, invasive MRSA infections are still rare. Between 2004 and 2006, 7 epidemic MRSA strain types were defined to be community-associated (Kanerva, et al., 2009). Of all 298 CA-MRSA isolates studied, 90 (30%) were PVL-positive. On account of the complexity of obtaining reliable information on the origin of MRSA, the Finnish Study Group for Antimicrobial Resistance (FiRe) has since 2005 collected data only on whether MRSA has been isolated from blood or other clinical samples. Since the beginning of the 2000s, new cases of MRSA have been encountered particularly in elderly persons and in long-term care facilities (Kerttula, et al., 2004; Laine, et al., 2012).

An LA-MRSA isolation frequency of 0.6% from major food animals was reported in 2003 from Korea where pigs were not found to be colonized at that time (Lee, 2003). After the recovery of a reservoir of LA-MRSA from pigs in the Netherlands (Voss, et al., 2005; van Cleef, et al., 2011), attention has been focused on a specific strain: MRSA ST398. In addition to the Netherlands, MRSA ST398 has been detected in pigs and cattle also in other countries including France, Denmark and the USA (Huijsdens, et al., 2006; Guardabassi, et al., 2007; Smith, et al., 2009). This suggests that MRSA ST398 might be of international importance as a pig-MRSA. Also in Finland, MRSA has been irregularly detected in pets and farm animals as well as in persons working with animals, although animal contact has not always been established (Salmenlinna, et al., 2010). MRSA ST398 was first recognized here in 2007, when it caused a veterinary hospital epidemic of 13 horses and 1 employee (Salmenlinna, et al., 2010). In 2009, EU Member States started a 1-year survey for LA-MRSA: among 59 finishing pig and 36 farrowing farms examined in Finland, 13 and 1 of these groups, respectively, were positive for MRSA (www.evira.fi). This results in an overall prevalence of 14.7%, which may not be an accurate figure owing to flaws in collecting samples for this survey. Anyway, the LA-MRSA prevalence on Finnish pig farms markedly exceeds the EU baseline prevalence.

Depending on regional livestock density, 0-25% of human MRSA isolates are associated with livestock. Human infections caused by LA-MRSA are rare and there is no evidence on its significant infiltration to healthcare systems (van den Broek, et al., 2009). Various characteristics of HA-, CA- and LA-MRSA are listed in Table 5.

**Table 5.** Methicillin-resistant *Staphylococcus aureus* (MRSA) epidemiology*

| Association | MLST CC[a] | SCC*mec*[b] | Characteristics | PVL[c] |
|---|---|---|---|---|
| HA-MRSA[d] | 5, 8, 22, 30, 45 | I-III | Beta-lactam resistance (SCC*mec* I)<br>Multiresistance (SCC*mec* II and III) | Rare |
| CA-MRSA[e] | 1, 8, 30, 59, 80, 93 | IV-V | Beta-lactam resistance | Common |
| LA-MRSA[f] | 398 | V | Multiresistance (SCC*mec* II and III) | Rare |

[a]MLST CC, multilocus sequence type, clonal cluster

[b]SCC*mec*, staphylococcal cassette chromosome *mec* type (I-V)

[c]PVL, expression of Panton-Valentine leucocidin associated with increased virulence

[d]HA-MRSA, healthcare-associated MRSA

[e]CA-MRSA, community-associated MRSA

[f]LA-MRSA, livestock-associated MRSA

*Modified from Kim, 2009 and from Monecke, et al., 2011

### 2.2.2.4. MRSA strains carrying the novel *mecC* gene

MRSA strains equipped with a new *mecA* homologue, the *mecC* ($mecA_{LGA251}$) gene were initially isolated from *S. aureus* LGA251 strains present in dairy cattle in England (García-Álvarez, et al., 2011). *MecC*-carrying MRSA strains are evidently globally distributed in animals and humans, and reports on their existence have already been published from England, Scotland, Denmark, Germany, Norway, Sweden, and France (García-Álvarez, et al., 2011; Laurent, et al., 2012; Petersen, et al., 2012). Retrospectively studied, these isolates appeared in Denmark in the beginning of the 2000s. In the period between 2003 and 2011, *mecC* was present in 1.5% (112/6920) of MRSA isolates, with an increasing tendency, reaching 1.9% (21/1097) in 2010 and 2.8% (36/1294) in 2011 (Petersen, et al., 2012). Epidemiological data on Danish *mecC*-positive MRSA strains from Denmark has shown that these microorganisms were primarily community-acquired (Petersen, et al., 2012). Affected persons were older than those with *mecA*-positive CA-MRSA strains. Moreover, these strains caused more human cases in rural areas as compared with the capital region, plausibly indicating that contact with livestock may be a risk factor predisposing a person for colonization

with a *mecC*-positive MRSA strain. *MecC*-carrying MRSA was found in human, bovine and ovine samples indicating the existence of many different reservoirs (Petersen, et al., 2012).

The clinical importance of *mecC*-positive MRSA strains is so far not definitely known. The detection of the *mecC* gene in MRSA isolated from blood and infected wounds suggests that these microorganisms are capable of causing clinical illness (García-Álvarez, et al., 2011). Whether the illness is equal in severity to that caused by *mecA*-positive MRSA is not clear before evidence from epidemiological and virulence investigations is obtained.

### 2.2.3. Antimicrobial treatment options

None of the beta-lactam antibiotics generally used in the first-line empiric treatment for severe infections is effective in MRSA infections. MRSA strains commonly have a MDR phenotype. Resistance to the fluoroquinoles, aminoglycosides and macrolides is widespread. The most important antimicrobial agents currently available for the treatment of MRSA infections, their main indications, and the specific aspects to be considered in a clinical setting are presented in Table 6.

For nearly half a century, glycopeptide antibiotics (vancomycin and teicoplanin) have been the mainstay for parenteral therapy of severe MRSA infections (Gould, et al., 2009; Liu, et al., 2011). Virtually all MRSA strains still remain susceptible to glycopeptides, and these agents are recommended as the first choice alternatives also for empiric treatment of suspected invasive infections in which MRSA is regarded as a potential causative agent. Therefore, the emerging resistance and decreasing susceptibility of MRSA to glycopeptides have been a major concern. The transfer of *van* resistance genes from *Enterococcus* species to *S. aureus* has been shown in *vitro* (Noble, et al., 1992), and also *in vivo* (Kacica & McDonald, 2004). It is fortunate that the few vancomycin-resistant *S. aureus* (VRSA) i.e. glycopeptide-resistant *S. aureus* (GRSA) strains so far identified have not led to secondary cases and that the responsible genetic elements have been unstable in staphylococci (Courvalin, 2006). More disquieting are the so-called vancomycin-intermediate *S. aureus* (VISA) i.e. glycopeptide-intermediate *S. aureus* (GISA) strains, in which the MICs of vancomycin are $\geq$ 4 mg/L and, also, strains with MICs of vancomycin $<$ 2 mg/L that exhibit heteroresistance (hVISA) (Moellering, 2012). Strains like that are often associated with therapeutic failure of glycopeptides (Howden, et al., 2004).

**Table 6.** Clinically the most important antimicrobial agents effective against methicillin-resistant *Staphylococcus aureus*, their key indications and comments on their use in a clinical setting*

| Agent | Route | Use as monotherapy | Key indications | Comments |
|---|---|---|---|---|
| Vancomycin | iv[a] | yes | bacteremia; serious soft-tissue infections; bone infection | slowly bactericidal; treatment failures reported despite minimal inhibitory concentrations in susceptible range; dose adjustment required in renal impairment; not orally absorbed; poorly predictable blood levels mean monitoring essential in serious infection |
| Teicoplanin | iv | yes | bacteremia; serious soft-tissue infections | slowly bactericidal; loading doses essential; dose adjustment required in renal impairment; not orally absorbed; poorly predictable blood levels mean monitoring essential in serious infection |
| Linezolid | iv or po[b] | yes | pneumonia; serious soft-tissue infection; VISA[c] (GISA)[d] and VRSA[e] (GRSA)[f] infections | bacteriostatic; oral form with excellent bioavailability; favorable lung pharmacokinetics; many treatment failures reported; according to manufactorer's recommendation therapy should be restricted to 28 days; oral form expensive |
| Daptomycin | iv | yes | bacteremia; right-sided endocarditis; serious skin and soft-tissue infections | bactericidal; inactivated by surfactant; cannot be used for pneumonia; elevations in creatinine phosphokinase possible; emergence of resistance reported; dose adjustment required in severe renal impairment |
| Tigecycline | iv | yes | skin and soft-tissue infections | bacteriostatic; excellent tissue penetration; serum levels may be inadequate to treat bacteremia; activity also against anaerobes and many gram-negative organisms |
| Clindamycin | iv or po | opinions differ whether can be used as a sole agent | skin and soft-tissue infections; bone and joint infections | bacteriostatic; excellent tissue penetration, especially in bone and abscesses; evidence of efficacy as a sole agent against strains with erythromycin resistance but risk of emergence of resistance exists |

| Agent | Route | Use as monotherapy | Key indications | Comments |
|---|---|---|---|---|
| Co-trimoxazole | iv or po | yes | skin and soft-tissue infections; eradication therapy in combination | bactericidal, limited information on use in severe MRSA infections |
| Trimethoprim | po | no | urinary tract infections; other use in combination therapy | bacteriostatic; limited information on use in MRSA infections |
| Tetracyclines | po | yes | skin and soft-tissue infections; eradication of carriage | bacteriostatic; emergence of resistance; limited information on use in severe MRSA infections |
| Fucidic acid | po | no, except in topical use | skin and soft-tissue infections; eradication of carriage | resistance – an emerging problem with systemic and topical use |
| Rifampicin | iv or po | never | bone and joint infections: skin and soft-tissue infections, adjunct therapy in prosthetic infections, e.g., joints and intravascular catheters | bactericidal; high intracellular levels; emergence of resistance during therapy a hazard; active against organisms in biofilms; should be used in combination with another active drug; role in staphylococcal infections controversial |

[a]iv, intravenous
[b]po, per oral
[c]VISA, vancomycin-intermediate *Staphylococcus aureus*
[d]GISA, glycopeptide-intermediate *Staphylococcus aureus*
[e]VRSA, vancomycin-resistant *Staphylococcus aureus*
[f]GRSA, glycopeptide-resistant *Staphylococcus aureus*
*Table modified from Cosgrove & Fowler, 2008; Gould, et al., 2009; and Liu, et al., 2011

New antimicrobial agents, such as linezolid and daptomycin, have been recently introduced to the treatment of MRSA infections. Basic data about these novel antibiotics are presented here in the text while Table 6 is referred to regarding data on older antimicrobial agents. Linezolid is a bacteriostatic agent effective against gram-positive cocci including MRSA (Ager & Gould, 2012). It has 100% oral bioavailability; thus, oral administration should be used whenever possible. Linezolid may be somewhat more effective than vancomycin for the treatment of MRSA pneumonia (Rubinstein, et al., 2008), but its superiority has so far not been definitely

proven (Liu, et al., 2011). Although some authorities have recommended the use of linezolid in bacteremia and endocarditis (Gould, et al., 2009), others have advised against it (Cosgrove & Fowler, 2008). This is in line with the recent guidelines given by the Infectious Diseases Society of America (IDSA) for the treatment of MRSA infections (Liu, et al., 2011). Following a familiar pattern *S. aureus* strains resistant to linezolid have already emerged (Morales, et al., 2010). It is of note that linezolid has exhibited *in vitro* activity also against VISA and VRSA strains (Wootton, et al., 2002).

Daptomycin is a rapidly bactericidal agent which is active against almost all gram-positive cocci, including MRSA. Daptomycin offers an acceptable alternative to glycopeptides for the treatment of bacteremic MRSA infections (Cosgrove & Fowler, 2008; Liu, et al., 2011). However, daptomycin should not be used for the treatment of pneumonia because its activity is inhibited by pulmonary surfactant (Gould, et al., 2009; Liu, et al., 2011). Resistance to daptomycin has been described to emerge during treatment (Yang, et al., 2010).

Tigecycline is also a novel broad-spectrum antibiotic effective against MRSA and, in addition, against anaerobes and many gram-negative bacteria (Townsend, et al., 2006). Tigecycline is a bacteriostatic agent endowed with a rapid and excellent tissue penetration (Townsend et al., 2006). This results in considerably low serum concentrations. Because of a recent warning from the United States Food and Drug Administration (FDA) indicating an increased risk in all-cause mortality with tigecycline vs. comparative drugs, tigecycline is not deemed a first-choice option in any indication, bearing in mind the availability of multiple other agents with activity against MRSA (Liu, et al., 2011). However, if a skin and soft-tissue infection is evidently polymicrobial (e.g., diabetic foot infection) and MRSA is considered to be an important pathogen, monotherapy with tigecycline might be an alternative (Gould, et al., 2009).

For outpatient care of mild CA-MRSA infections oral antimicrobial treatment is commonly sufficient, although many patients with drainable lesions are cured with surgical drainage alone. CA-MRSA strains differ from HA-MRSA strains in their susceptibility to different antimicrobial groups. CA-MRSA strains are usually susceptible to co-trimoxazole, rifampicin, and gentamycin, and most of them are also susceptible to clindamycin (King, et al., 2006). In addition, CA-MRSA strains are often susceptible to tetracyclins. In contrast, resistance to macrolides and fluoroquinolones is common (King, et al., 2006; Kanerva, et al., 2009). Clindamycin, co-trimoxazole, and tetracyclins have been the most commonly used oral antimicrobials for the treatment of CA-MRSA infections. It has been advised that for a disease believed to be caused by MRSA producing PVL, treatment with either linezolid or clindamycin should be considered since both of them are known to modify the expression of PVL *in vitro* (Stevens, et al., 2007; Gould, et al., 2009).

### 2.2.4. Eradication of carriage

Different approaches for eradication of MRSA colonization have been described. In a systematic review of 23 clinical trials, short-term (4-7 days) topical nasal application of mupirocin was found the most effective treatment with an estimated success probability of 90% 1 week after treatment and 60% after a longer follow-up period, ranging from 14 days through 365 days in different studies (Ammerlaan, et al., 2009). The estimated eradication rate with oral antibiotics was 60% 1 week after treatment and 50% after longer follow-up periods. MRSA commonly colonizes extra-nasal sites, which reduces the efficacy of intranasal mupirocin treatment. Although systemic therapy is used for eradication treatment, it should only be considered in patients with multiple factors known to be associated with treatment failure (e.g., skin lesions, mupirocin-resistant strains, foreign bodies, and extra-nasal colonization sites, especially the throat) in cases where eradication of MRSA carriage is estimated to be important for the patient (Coia, et al., 2006; Ammerlaan, et al., 2009). Some carriers are cleared of MRSA spontaneously. The half-life of MRSA carriage without eradication therapy has been reported as 40 months (Sanford, et al., 1994).

### 2.2.5. Control and prevention of MRSA

#### 2.2.5.1. Measures to limit the spread of MRSA

MRSA is spread by direct contact with MRSA-positive individuals, and the control measures are aimed at cutting off routes of transmission. The main control measures include 1) MRSA screening, 2) staff hand hygiene, 3) isolation or cohorting of MRSA-positive patients, and 4) decolonization of MRSA carriers. In addition, surveillance, environmental cleaning, antimicrobial stewardship, and education of the staff play central roles in various strategies. Stringent hand hygiene of the hospital personnel is of utmost importance to restrict the dispersal of nosocomial microorganisms from patient to patient, and between patients and staff. Decolonization strategies tend to reduce the MRSA load in hospitals. Although complete or permanent eradication of MRSA from a colonized patient is often not possible, even a decrease in carriage can diminish the risk of transmission of MRSA to other patients, as well as the risk of inoculation of MRSA into the patient's own surgical wounds during the hospital stay (Coia, et al., 2006). Surveillance is routine as part of the hospital's infection control program. Antimicrobial stewardship addresses avoidance of inappropriate or excessive antibiotic therapy, with a special aim to restrict the use of glycopeptides only to the necessary. Education of staff is essential to establish adequate professional skills combined with motivation and devotion to exercising impeccable hospital hygiene. One of the reasons for the relative lack of success in controlling the spread of MRSA in many countries may be inadequate resources, but also the failure of healthcare professionals to comply with good infection control practice manifested as poor adherence to isolation

precautions and hand hygiene. Of the measures listed above, the topic of screening is presented below in more detail.

#### 2.2.5.2. Screening for MRSA colonization

Active screening of patients for MRSA colonization is a foundation for the containment of MRSA in hospitals. Screening is not a control measure in itself, but it provides data for a targeted approach to direct the interventions to appropriate patients (Coia, et al., 2006). There is no all-inclusive guidance from the literature on which patients should be screened. It seems reasonable to screen on admission such patients which are considered to be at a high risk for carriage of MRSA. They include those who have been a) infected or colonized with MRSA in the past, b) in contact with previously recognized MRSA carriers, and c) recent inpatients at hospitals abroad or in hospitals known to have an MRSA problem (Coia, et al., 2006). Depending on the local epidemiology of MRSA in a country, region, or hospital, additional risk groups may be defined.

When a new case of MRSA is found on a hospital ward, at least all recognized contact patients should be screened. When 2 or more new cases of MRSA are found, indicating that MRSA has been transmitted on the ward, screening of all other patients is indicated (MRSA-asiantuntijaryhmän suositus, 2004, available at http://www.thl.fi). Staff screening is usually not recommended routinely.

If possible, screening for MRSA should be directed at common sites of MRSA carriage (nose, perineum, throat, axillae/groin) and, in addition, at skin lesions and wounds, sites of insertion of intravenous catheters, and catheter urine (Coia, et al., 2006). Screening samples may not become positive immediately after the patient has been exposed to MRSA. In such cases, more than 1 set of screening samples during e.g., a 1-week period may be required. There is an abundance of screening samples to be examined even during small outbreaks of MRSA. Thus, high-throughput screening tools are needed. Rapid methods for screening are also indispensable, given that the risk patients are nursed in contact isolation while waiting for the screening results, which may take up to 72 h by conventional culture-based methods. Calculated by mathematical modelling, the use of rapid tests to detect nasal carriage proved more effective in low-prevalence settings, where this tactic can potentially reduce isolation requirements by > 90%, whereas in high-endemicity settings isolation requirements are reduced by only 20% (Bootsma, et al., 2006).

#### 2.2.5.3. Various national MRSA guidelines

National guidelines on how to control the spread of MRSA and to prevent the development of MRSA infections are available in many countries (Humphreys, 2007). These guidelines have usually been drafted by national agencies or well-known expert organizations. Many of the recommendations of the earlier guidelines (Report of a

Combined Working Party of the Hospital Infection Society and British Society for Antimicrobial Chemotherapy, 1986; Working Party Report, 1990; MRSA-asiantuntijaryhmän suositus, 1995) were not based on strong scientific evidence, i.e., on well-designed, experimental, clinical, or epidemiological studies. Nevertheless, they relied on common sense, professional experience, and clear logic of recommended implementations, and were often met with general approval. Recent national MRSA guidelines (Coia, et al., 2006; Rebmann & Aureden, 2011b) are more often substantiated by research data, but there are still important areas in which scientific evidence is missing. All guidelines need to be adjusted for local use considering many practical issues, e.g., the prevalence of MRSA, isolation facilities, and nursing staff levels. Modifications are also required when MRSA control measures are applied in long-term facilities (Rebmann & Aureden, 2011a).

In countries where the rates of MRSA are very low and MRSA is not endemic, strict control measures are recommendable whenever MRSA is introduced into a hospital. In the Netherlands and in the Nordic countries the incidence of MRSA infection and colonization is still on a low level. These countries are characterized by implementation of firm national guidelines for the control of multiresistant *S. aureus* strains. In the Netherlands a stringent "search and destroy" policy for the prevention of MRSA was elaborated as a Dutch national strategy, and has been followed since 1988 (Dutch Working Party on Infection Prevention, WIP, 2003). The Dutch "search and destroy" policy focuses on (1) defining groups at risk and screening of both patients and healthcare staff at risk, (2) strict isolation of MRSA-positive patients and patients considered to be at risk pending culture results, (3) outbreak management and, after hospital discharge, (4) follow-up of carriers and (5) elimination of carriage if feasible (Vos, et al., 2009; Dutch guidelines, Infection Prevention Working Party, revision 2012, available at: www.wip.nl/UK/free_content/Richtlijnen/MRSA%20hospital.pdf). In brief, strict isolation in the Dutch context means that a patient is cared for in a single room with a closed door and an own bathroom, preferably with an anteroom and regulated negative air pressure. The attending healthcare personnel wear masks, gloves, gowns with long sleeves and cuffs, and caps upon entering the isolation room. Careful disinfection of the hands is required after removing the protective equipment. The room is disinfected twice daily (Vos, et al., 2009).

In Finland, all patients recognized as MRSA-positive should be nursed in hospital following contact isolation precautions (MRSA-asiantuntijaryhmän suositus, 2004). To sum up, an MRSA-positive patient is cared for in a single room with a closed door and an own bathroom, or in a cohort of MRSA-positive patients. An anteroom is recommended, being crucial if the patient is a heavy MRSA disperser. The hospital personnel wear gloves whenever an isolation room is entered. Gowns or plastic aprons are necessary at least when attending to a patient, or if a physical contact is otherwise considered possible. Surgical masks are worn e.g., when MRSA-positive wounds are tended, or when a nasal or throat carrier has a respiratory infection.

Decolonization of MRSA-positive patients is not a common method in Finland to decrease the MRSA load in hospital. Indications for decolonization treatment are always carefully considered. Decolonization may be prudent in longterm care patients in non-endemic healthcare facilities, in patients with long hospital stays or before extensive surgical procedures (MRSA-asiantuntijaryhmän suositus, 2004). Also MRSA-colonized hospital and healthcare personnel usually undergo decolonization treatment. There is no international consensus as to when a carrier can be deemed to be cleared of MRSA. According to the Finnish guidelines, it is possible to discontinue contact isolation precautions during hospital stay, if 3 sets of screening samples from various body sites taken at 1-week intervals (collected ≥ 1 week after decolonization therapy) have proven MRSA-free. It is important to remember that the samples may turn positive again later, especially during antimicrobial treatment.

Reports from the Netherlands and Finland show that controlling or even eliminating MRSA is possible, if strict measures are taken before the organism becomes endemic (Vandenbroucke-Grauls, et al., 1991; Kotilainen, et al., 2001; Kotilainen, et al., 2003; Pastila, et al., 2004; van Trijp, et al., 2007). In contrast, in countries and hospitals with endemic MRSA, elimination is not easily possible, and the main aim is usually to suppress the microorganism i.e. to reduce the number of patients becoming colonized with MRSA, and to limit severe damage caused by MRSA (Humphreys, 2007). The measures for the containment of MRSA are quite similar in high- and low-prevalence hospitals but the capacity to comply with the recommendations may be different. In low-prevalence settings firm adherence to the required measures is realistic, whereas in hospitals with endemic MRSA it may be sensible to focus screening and control efforts mainly on high-risk units. When mathematical models were applied to quantify the effectiveness of different infection control measures in various environments, any implementation included in the "search and destroy" policy would reduce endemic prevalence (Bootsma, et al., 2006). Isolation of MRSA-positive patients indentified by clinical cultures as the only intervention may reduce the endemic prevalence to 5% within 15 years, when an isolation efficacy is assumed to be 100%. On the other hand, the application of the complete "search and destroy" policy would reduce endemic prevalence to < 1% within 6-12 years (Bootsma, et al., 2006).

#### 2.2.5.4. Containment of CA-MRSA

The ongoing change in MRSA epidemiology is considered a threat as the above measures targeted at controlling the spread of MRSA in hospitals are not without difficulty adapted to the control of MRSA in the community (Stefani, et al., 2012). Recently, an update on the prevention and control of CA-MRSA was summarized by esteemed international authorities (Skov, et al., 2012). It was concluded that preventing the dissemination of these microorganisms throughout the general population requires a multifaceted approach, including common hygiene and cleaning measures, antibiotic stewardship, screening, and decolonization of proven carriers in selected (or outbreak) situations.

### 2.2.6. Reasons for containment of MRSA

The reasons for continuing efforts to control MRSA are still considered valid by most infection control experts. Nevertheless, justification for not implementing specific measures has been argued by others (Lacey, 1987; Barrett, et al., 1998). It has been questioned whether controlling this microbe is reasonable, feasible, or even justified (Bowler, 1997; Barrett, et al., 1998). This can be answered by pointing out a number of sound reasons arguing for the relevance of containment of MRSA.

Considering individual patients, contagion with MRSA may be only transient but may also lead to prolonged carriage which is difficult, if not impossible, to eradicate with the present-day decolonization systems. Although a majority of MRSA-positive patients are colonized with no clinical symptoms, a certain proportion of them will develop infections which may be invasive, or even result in death. For some reason, patients colonized with MRSA have been reported to be 4 times more likely to develop an invasive infection than those colonized with MSSA (Safdar & Bradley, 2008). There has been plenty of discourse during the years as to whether the prognosis of patients with MRSA bacteremia is better or worse than that for the patients with MSSA bacteremia. Various studies, including 1 meta-analysis, have shown that MRSA bacteremia is connected with a significantly higher mortality rate and length of hospital stay compared with MSSA bacteremia (Romero-Vivas, et al., 1995; Cosgrove, et al., 2003; Shurland, et al., 2007). However, also the opposite has been presented (Fowler, et al., 2003; Wang, et al., 2008). Further studies are evidently needed to resolve this important issue. The *mecA* gene is not a virulence factor in itself, and MRSA strains are not generally considered more virulent than MRSA strains. However, there may be a delay in the commencement of adequate antimicrobial treatment in MRSA infections. Undeniably, also the selection of effective antimicrobial agents in MRSA infections is more limited, and appropriate antibiotics are often more toxic or expensive than those used to treat MSSA infections.

Several economic evaluations have focused on the cost-effectiveness of MRSA control measures. A retrospective cohort study of patients with MRSA bacteremia that allowed for confounding variables has shown that patients with MRSA have a 1.5-fold longer duration of hospital stay and a 2-fold increase in hospitalization costs compared to patients with MSSA (Lodise & McKinnon, 2005). Other studies have indicated that screening programs resulting in implementation of MRSA control measures may lead to considerable cost-savings in healthcare facilities (Wernitz, et al., 2005; Nulens, et al., 2008). One should also consider the savings of additional costs by MRSA to patients and their families (e.g., loss of income), and to the society (e.g., absence from workforce). On the other hand, every new case of MRSA means significant costs for the institution. Costs of an outbreak of MRSA in a 1752-bed tertiary care hospital were analyzed in Finland a few years ago (Kanerva, et al., 2007). Extra costs were caused by

taking methicillin resistance into account in the treatment of recognized MRSA infections and loss of income owing to closed beds. Of the additional costs, 70% were caused by screening and 25% by contact isolation precautions. The authors conclude that the high costs of MRSA screening call for a thorough evaluation of the cost-benefit of the screening methods used.

Finally, it is vital to keep in mind that an unsuccessful control of an MRSA epidemic may result in an endemic situation. Endemic MRSA has a major impact on the use of antimicrobial agents in the hospital as well as on the antimicrobial policy applied. In endemic situations the usage of glycopeptides inevitably increases, since severe infections caused by MRSA are usually treated with vancomycin. This on its part increases the risk for the development of vancomycin-resistant gram-positive cocci including vancomycin-resistant enterococci (VRE) and vancomycin-nonsusceptible staphylococci (VISA, VRSA). To prevent the spread of vancomycin resistance the empiric use of glycopeptides has been discouraged already for years (HICPAC, the Hospital Infection Control Practices Advisory Committee, 1995). However, at some threshold of MRSA prevalence glycopeptides must be included in the empiric treatment of suspected sepsis (Gould, et al., 2009). The British authorities have proposed that the resistance rate could be 10% (Gould, et al., 2009). Today, control of MRSA may be more necessary than ever because of emergence of clinically significant *S. aureus* strains with reduced susceptibility to glycopeptides.

## 2.3. Laboratory diagnosis of MRSA

### 2.3.1. Identification of MRSA from pure cultured colonies

#### 2.3.1.1. Genotypic methods

##### *2.2.1.1.1. The gold standard: detection of the mecA gene*

An intact *mecA* gene is essential for the expression of clinically significant methicillin resistance in *S. aureus* (de Lencastre, et al., 1994). There are several auxiliary genes, found in both susceptible and resistant *S. aureus* strains, with effects on the level of methicillin resistance (de Lencastre & Tomasz, 1994). As a result, *S. aureus* strains possessing the *mecA* gene have variable resistance phenotypes from borderline to highly resistant. The heterogeneous nature of methicillin resistance limits the accuracy of all phenotypic methods; consequently, a genotypic approach, detection of the *mecA* gene, is accepted as the gold standard of the MRSA diagnostics (Chambers, 1997). Currently, a definite identification of methicillin resistance in any *S. aureus* strain can only be made based on the presence of the *mecA* gene.

Standard molecular assays using the *mecA* gene as a single target require isolation and identification of *S. aureus* by the common phenotypic methods before testing for methicillin resistance is possible. Methicillin-resistant CoNS commonly present in clinical samples also carry the *mecA* gene and would otherwise cause false-positive test results (Huletsky, et al., 2004). Thus, these assays are mainly used for confirmation only.

A new variant of the *mecA* gene (*mecC*), undetectable by the current gold standard PCR assays, was recently described (García-Álvarez, et al., 2011). It resides on a novel mobile staphylococcal cassette chromosome *mec* called type XI (Shore, et al., 2011). The protein has < 63% amino acid identity with PBP2a encoded by *mecA*, and was described in *S. aureus* and CoNS strains.

#### 2.3.1.2. Phenotypic methods

##### *2.3.1.2.1. Disk diffusion tests*

After identification of *S. aureus* from a pure culture, phenotypic methods can be used for the testing of methicillin resistance. Regarding *S. aureus* identification, it should be remembered that many MRSA strains fail to produce agglutination in the first-generation latex agglutination tests. These strains have been found to possess a polysaccharide capsule, which is thought to physically mask other cell surface components (Fournier, et al., 1989). Capsular types 5 and 8 predominate among clinical isolates (Hochkeppel, et al., 1987).

The disk diffusion method is the most widely used technique for susceptibility testing in clinical laboratories since it is inexpensive and technically simple. Interpretation of the test results has been well standardized. Both the US Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) periodically review the test method for optimal performance. Since 2011, the EUCAST standard is universally used in Finland. Isolated colonies from a pure culture are used to prepare an inoculum which is then applied on a Mueller-Hinton agar (MHA) plate. Paper disks containing fixed concentrations of antibiotics are placed on the inoculated plate surface. After 16 to 24 h of incubation visible zones of growth inhibition are formed around the discs. Zone diameters are interpreted using the CLSI/EUCAST criteria and the results are reported qualitatively as sensitive, intermediate, or resistant.

Expression of the *mecA* gene is affected by environmental factors such as temperature, osmolarity and certain antimicrobial agents (Ryffel, et al., 1994). Therefore, culture conditions are modified to enhance the expression of methicillin resistance. Previously, the use of 1 µg oxacillin disks and incubation at 35°C on MHA plates supplemented

with 2% NaCl were recommended by the CLSI for the testing of staphylococci for methicillin resistance. Recent studies suggest that disc diffusion techniques using cefoxitin are superior to oxacillin-based methods (Felten, et al., 2002; Swenson & Tenover, 2005). Cefoxitin has been recognized as a potent inductor of the *mecA* regulatory system (McKinney, et al., 2001). Two studies have shown improved accuracy of the cefoxitin disc diffusion test when performed at 30°C, although high accuracies in standard 37°C have been reported as well (Felten, et al., 2002; Cauwelier, et al., 2004). The EUCAST/CLSI standards currently promote usage of cefoxitin instead of oxacillin for MRSA diagnostics using the disk diffusion method (CLSI, 2009). For a 30 µg cefoxitin disk a single breakpoint is recommended: an inhibition zone diameter ≤ 21 mm (CLSI) / < 22 mm (EUCAST) indicates MRSA. MHA plates without additional salt and incubation at 35°C for 18 h should be used.

*2.3.1.2.2. Antimicrobial gradient method*

An Etest (AB Biodisk, Solna, Sweden) is the most commonly used antimicrobial gradient diffusion method. It employs a test strip impregnated with a dried antibiotic concentration gradient marked on the surface of a scale. Multiple strips can be placed on the surface of an agar plate that has been inoculated with a standard bacterial suspension. Again, a pure culture is needed before the Etest can be employed. After an overnight incubation the MIC can be determined by reading the scale on the strip at the intersection of the bacterial growth (Jorgensen & Ferraro, 2009). The Etest is a flexible and easy method that suits even smaller laboratories. The cost per strip is a few euros, which is multiplied by the number of different drugs tested. With certain combinations of organisms and antimicrobial agents, for example MRSA and vancomycin, the Etest tends to be biased (Prakash, et al., 2008).

Methicillin and oxacillin Etests can be used as a simple way to detect MRSA. According to CLSI, the MIC breakpoint of oxacillin resistance for *S. aureus* is ≥ 4 mg/L (CLSI 2009) which is in line with EUCAST, whose oxacillin MIC breakpoint is > 2 mg/L (http://www.eucast.org). A cefoxitin MIC value of ≥ 8 mg/L (CLSI) / > 4 mg/L (EUCAST) indicates MRSA. MHA plates supplemented with 2% NaCl and incubated at +35°C for 24 h are recommended (Weller, et al., 1997). The Etest is also widely used in further antimicrobial susceptibility testing of MRSA findings.

*2.3.1.2.3. Broth dilution*

The tube-dilution method was one of the earliest antimicrobial susceptibility testing methods. This involved preparing multiple dilutions of an antibiotic in a liquid growth medium. Separate test tubes for each dilution were all inoculated with standard bacterial suspension derived from the pure culture of the original clinical sample.

After an overnight incubation the tubes were examined for turbidity caused by bacterial growth. The lowest concentration that prevented growth was reported as the MIC value (Jorgensen & Ferraro, 2009).

In recent years the tube-dilution test has evolved into a more convenient microdilution procedure utilizing preprepared disposable microdilution trays and extensive automation. In a current assay the sensitivity to 12 antibiotics can be tested on a 96-well tray in a range of 8 two-fold dilutions (Jorgensen, 2007). Computerized reports are generated if an automated panel reader is used. Increasing turbidity is an unspecific sign of any bacterial growth. Prior isolation of the organism to be tested is required.

Several rapid automated antimicrobial susceptibility testing systems for *S. aureus* are based on a microdilution approach with sensitive, yet non-specific detection of bacterial growth in various antibiotic concentrations. The detection techniques include turbido-, fluoro- and colorimetric approaches. Four such devices have been approved to date by the FDA: the Vitek 2 (bioMérieux, Marcy l'Etoile, France), the MicroScan WalkAway (Siemens Healthcare Diagnostics, West Sacramento, CA, USA), the BD Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, Md, USA) and the Sensititre ARIS 2X (Trek Diagnostic Systems). Test results are commonly available within working hours and MICs for several different antimicrobial agents can be simultaneously tested. There is evidence that rapid susceptibility test results generated by these systems lead to financial and clinical benefits (Barenfanger, et al., 1999). The BBL Chrystal MRSA ID is based on monitoring oxygen consumption, which is increased in the presence of any growing aerobic bacteria (Becton Dickinson, Cockeysville, Md, USA). The test promises correct identification of MRSA within only 4 h (Qadri, et al., 1994). However, all of the rapid automated techniques described here still require an isolated organism for a starting point. A pure culture typically takes 18 to 24 h with an additional delay from identification of *S. aureus*.

*2.3.1.2.4. MRSA Screen*

The MRSA screen (Denka Seiken, Tokyo, Japan) latex agglutination test detects PBP 2a with a monoclonal antibody. The test is simple, accurate and requires only 15 min to differentiate between MRSA and MSSA colonies isolated on agar plates (Cavassini, et al., 1999). The test should only be applied after identification of the colony to the species level to rule out a false-positive test result by CoNS (van Leeuwen, et al., 1999).

### 2.3.2. Methods for screening of MRSA from nonsterile samples

#### 2.3.2.1. Sampling and enrichment

The anterior nares are considered the predominant site of MRSA carriage. However, 24% of MRSA-colonized patients show exclusive groin, rectal or perineal carriage. This will not be detected if only nasal samples are tested (Zhang, et al., 2007). Thus, multiple sampling sites should be included in any screening procedure. Fiber swabs are known to release only a small portion of organisms sampled onto solid media (Human & Jones, 2006). A flocked nylon swab (ESwab, Copan, Italy) transported in 1 ml of liquid medium has been demonstrated to improve the MRSA detection rate from colonization samples by 10% (Jones, et al., 2011).

Clinical MRSA colonization samples collected with swabs are commonly subjected to an overnight preincubation in a liquid medium. An initial enrichment step instead of primary plating is known to improve the MRSA recovery rate, as the amount of metabolically active bacteria in a colonization sample may be low (Davies & Zadik, 1997). On the downside, an extra day of incubation is required. Tryptic soy broth (TSB) or brain heart infusion (BHI) mediums supplemented with various concentrations of NaCl are commonly used (Safdar, et al., 2003; Karabiber, et al., 2009). Preincubation is followed by plating on solid media and another overnight incubation before isolated colonies are available for identification and antimicrobial susceptibility testing by the methods described above.

#### 2.3.2.2. Selective agars

Several selective and differential agars have been developed for MRSA screening purposes. They provide an attractive all-in-one solution without the high costs associated with molecular methods. The currently available chromogenic media include ChromID (bioMérieux, Marcy l'Etoile, France), MRSA Select (Bio-Rad Laboratories, Gent, Belgium), CHROMagar MRSA (CHROMagar Microbiology, Paris, France; BD Diagnostics, Erembodegem, Belgium), Chromogenic MRSA/Denim Blue agar (Oxoid, Basingstoke, UK), oxacillin resistance screening agar base (ORSAB; Oxoid), MRSA Ident agar (Heipha GmbH, Eppelheim, Germany), and Chromogen oxacillin *S. aureus* medium (Axon Labs AG, Stuttgart, Germany). Each contains various antibiotics and an indicator that changes color as a result of *S. aureus* metabolic by-products. Thus, growth of the majority of contaminating microbes is inhibited while MRSA isolates form colonies of a characteristic color. The disk diffusion test is well suited for further studies of isolates obtained from selective agars.

Selective chromogenic agars can be applied to direct plating, although the sensitivity is significantly increased by the addition of an enrichment step (Böcher, et al., 2008). Most selective chromogenic media containing cefoxitin, such as the CHROMagar, are

highly specific (Wendt, et al., 2010): colonies of typical mauve color and morphology after 24 h of incubation can be reported as MRSA without confirmation. However, widely varying sensitivities between media and incubation times in MRSA screening have been reported (Malhotra-Kumar, et al., 2008). The CHROMagar sensitivity of 67% after 24 h of incubation was found better than the sensitivities of ChromID or ORSAB (Compernolle, et al., 2007). Prolonging the incubation improves the sensitivity while the specificity is adversely affected.

#### 2.3.2.3. Direct detection of MRSA from colonization samples

##### *2.3.2.3.1. Rapid PCR techniques with multiple primers*

Real-time PCR assays with multiple target sequences have been developed to allow detection of MRSA directly from nonsterile clinical samples. Differentiation of *mecA*-positive *S. aureus* from *mecA*-positive CoNS is possible by amplification of a sequence covering the *SCCmec* (carrying *mecA*) and the adjacent chromosomal species-specific sequence (*orfX*). Multiple primers are needed to cover the different *SCCmec* variants. Currently available rapid PCR-based molecular MRSA tests targeting the SCC*mec*-*orfX* junction include the IDI-MRSA assay (GenOhm, San Diego, CA, USA; BD Diagnostics), the GeneXpert MRSA assay (Cepheid, Sunnyvale, CA, USA), and the GenoType MRSA direct assay (Hain Lifescience, Nehren, Germany). The LightCycler Staphylococcus and MRSA detection kit (LC assay; Roche Diagnostics, Mannheim, Germany), and the Hyplex StaphyloResist PCR (BAG, Lich, Germany) detect unlinked target sequences and are considered inferior in performance (Malhotra-Kumar, et al., 2008).

The IDI-MRSA Kit detects MRSA from a nasal swab in less than an hour (Huletsky, et al., 2004). Excellent 96% sensitivity and 96% specificity was initially reported for the assay. However, outside a research laboratory setting the assay sensitivity soon dropped to an unacceptable level of 60% (Desjardins, et al., 2006). Significantly reduced sensitivity has later been associated with certain epidemiological environments (Sissonen, et al., 2009). Strains in which methicillin resistance is based on the presence of the *mecC* gene remain undetected by this assay (García-Álvarez, et al., 2011).

A low positive predictive value (PPV; 65.9%) for the IDI-MRSA test due to MSSA strains carrying non-functional, "empty cassette" *SCCmec* has been reported (Stamper, et al., 2011). A high prevalence of false-positive test results has also been associated with the other assays targeting the SCC*mec*-*orfX* junction (Arbefeville, et al., 2011). The PPV of the Hyplex StaphyloResist assay was only 31.4% (Daeschlein, et al., 2006). Consequently, verification of all rapid PCR-positive samples by conventional phenotypic methods has been suggested (Stamper, et al., 2011; Arbefeville, et al., 2011).

*2.3.2.3.2. BacLite Rapid assay*

The BacLite Rapid (3M Company, Maplewood, MN, USA) assay allows the presence or absence of MRSA to be determined directly from a clinical specimen within 5 h (Johnson, et al., 2006). Unlike the other rapid phenotypic techniques, no time-consuming steps for prior isolation are needed. The test utilizes a culture-based approach with successive semiautomated selectivity steps. These include selective enrichment culture in the presence of cefoxitin and colistin, an immunomagnetic extraction step, selective lysis and, finally, detection of adenylate kinase activity (von Eiff, et al., 2008). A study with nasal colonization samples showed 90.4% sensitivity and 95.7% specificity (Johnson, et al., 2006). Cost per analysis is considerably lower than with most commercial molecular tests. Unfortunately, especially some CA-MRSA are likely to be missed by this sophisticated assay because ciprofloxacin-susceptible MRSA cannot be detected. The lysis step prevents any further conventional post-analysis of the sample.

## 2.4. Two-photon excited fluorescence detection (TPX) technology

### 2.4.1. Two-photon excited fluorescence

Two-photon excitation of fluorescence is a phenomenon of non-linear optics that takes place when 2 photons are simultaneously (less than $10^{-15}$s apart) absorbed by a single molecule. A 2-photon excitable fluorescent molecule is then excited as if a single photon twice the energy had been absorbed. When the molecule relaxes back to the ground state a high-energy photon is emitted (Figure 2). Thus, 2-photon excited fluorescence is of higher energy (i.e. higher frequency and shorter wavelength) than the original excitation light. The intensity of 2-photon excited fluorescence generated is proportional to the square of the power of the excitation light.

The theory of 2-photon excitation was first introduced by Maria Göppert-Mayer as early as in 1931 (Göppert-Mayer, 1931). However, there were no practical applications of the principle for decades. It was not until the 1960s when high-intensity laser light sources became available that measurable quantities of 2-photon excited fluorescence could be generated. Adoption of pulsed light sources further increase generation of 2-photon excited fluorescence over that of continuous excitation at the same average power (Hänninen, et al., 1994). Use of 2-photon excited fluorescence in high-resolution biomedical imaging was first described in 1990 (Denk, et al., 1990). The early applications relied on pulsed high-intensity infrared lasers with ultrashort pulse widths in the femtosecond range. However, the high costs and bulky instrumentation of these devices rendered them impractical outside research laboratory settings.

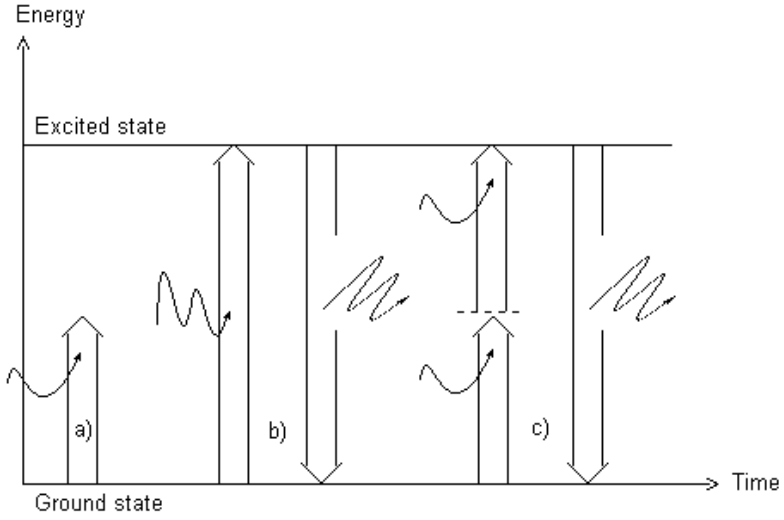


FIGURE 2. a) A single photon of insufficient energy to excite the molecule, b) a high energy photon excites the molecule and a photon of similar energy is emitted when the excited state is relaxed, c) 2 photons each of half the required energy may excite the molecule if simultaneously absorbed.

### 2.4.2. TPX microfluorometer

In the laboratory of Biophysics, University of Turku, Turku, Finland, research on 2-photon excitation of fluorescence has been carried out since the 1990s. By increasing the measurement time and by some innovative optical arrangements small and inexpensive near-infrared semiconductor lasers could be employed in biomedical applications of 2-photon excitation (Hänninen, et al., 2000). The simplified optical arrangement utilized in a TPX microfluorometer is presented in Figure 2 (Soini, 2002).

Semiconductor lasers utilized in the TPX technology typically produce pulses with pico- to nanosecond duration. Custom-made tools were developed for characterization and trouble-shooting of these lasers (Stenholm, et al., 2004).

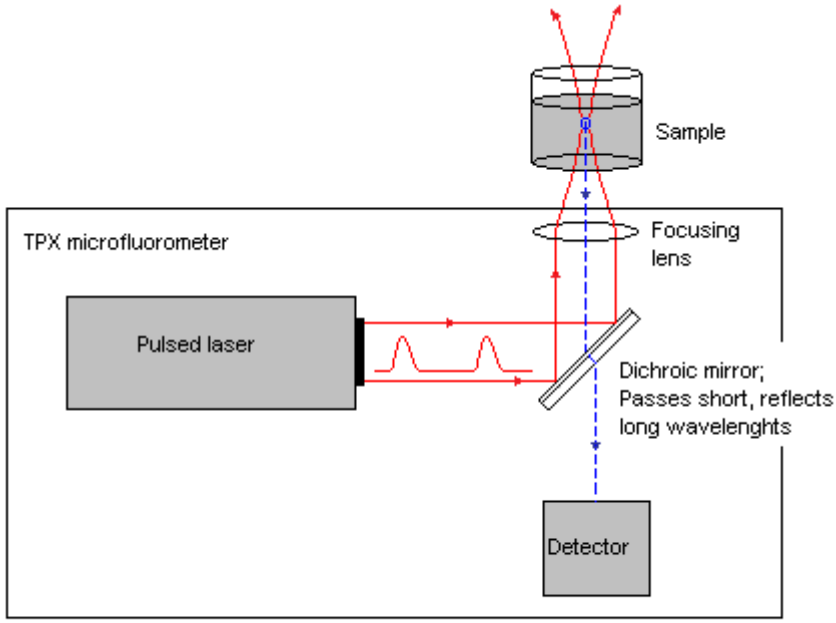


FIGURE 3. Optical arrangements of a 2-photon excited fluorescence detection (TPX) microfluorometer (modified from Soini, 2002)

Within the sample volume a sufficient intensity for generation of 2-photon excited fluorescence is only achieved within the point like focal volume (1 fl) of the laser beam (Figure 3). Scattering of excitation light and generation of single-photon excited fluorescence takes place in all parts of the laser beam path. Both have intensities orders of magnitude higher than the 2-photon excited fluorescence. However, the much shorter wavelength of 2-photon excited fluorescence allows it to be effectively filtered out by a simple dichroic mirror (Figure 4).

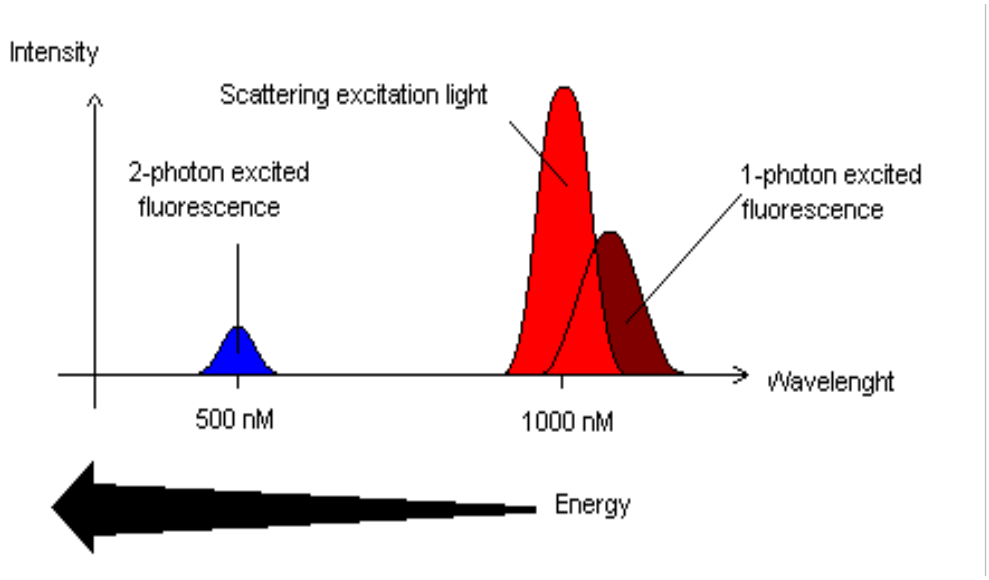


FIGURE 4. Two-photon excited light generated within the laser beam focus is of short wavelength and can be optically isolated from scattering excitation light and single photon excited fluorescence originating from outside the focal volume.

In an immunometric TPX assay polystyrene microparticles a few micrometers in diameter are utilized as solid phase carriers for sandwich-type immunocomplex formation. The plastic walls of the sample cuvette itself serve as a basis for immunocomplex formation in standard enzyme-linked immunosorbent assays (ELISA). The microparticles are passively coated with a capture antibody while fluorescently labeled tracer antibodies are also added into the reagent mixture. Both the tracer and the capture antibodies have a high affinity to the analyte. As a result of specific antibody binding reactions fluorescent molecules are concentrated on microparticle surfaces in proportion to the analyte concentration (Figure 5).

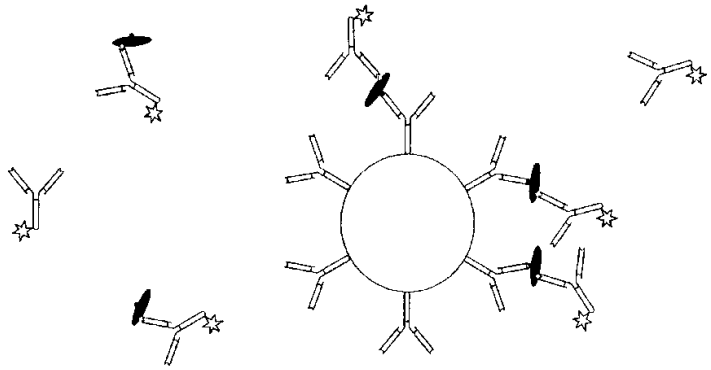

FIGURE 5. The principle of the 2-photon excited fluorescence detection (TPX) immunometric assay. The reagent mixture contains fluorescently labeled tracer antibodies and microparticles coated with capture antibodies. The fluorophores (star) are bound on microparticle surfaces via analyte molecules (black ovals) (Soini, 2002).

The intensity of the scattering excitation light is increased when a microparticle in the sample solution coincides with the path of the laser beam. The polystyrene microparticle presents a reflective surface to the beam. The closer to the focus the particle comes, the higher the intensity of the scattering. Measurement of 2-photon excited fluorescence is only activated when a certain threshold value of scattering excitation light is exceeded, indicating the presence of a microparticle within the focal volume of the laser beam. This arrangement allows the measurement of fluorescence from an individual microparticle at a time. The principle is presented in Figure 6.

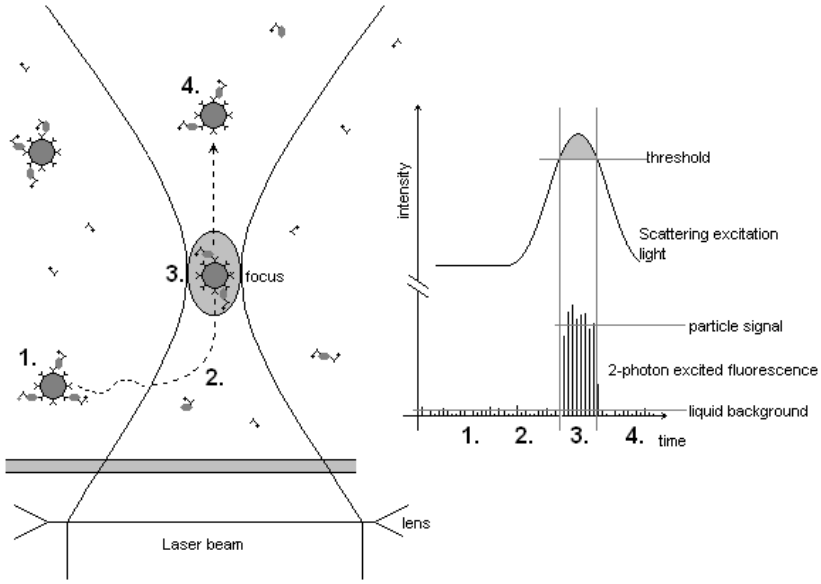


FIGURE 6. Intensity of scattering excitation light is increased when a microparticle comes into focus, triggering the measurement of 2-photon excited fluorescence (particle signal). The liquid background signal is generated by random/unbound tracer molecules within the focal volume.

Optical separation of the microparticles offers several advantages over conventional methods such as ELISA. There is no need to wash out the unbound fraction of fluorescent tracer molecules. Consequently, a sample can be repeatedly measured without the need to interrupt ongoing reactions within. Even continuous monitoring is possible. Sample volume does not affect the signal level either.

Quantitative TPX immunometric assays share the same sensitivity and basic kinetics with standard ELISA assays. At analyte concentrations below the assay detection limit the TPX fluorescence signal remains at background level. Within the assay dynamic range a direct correlation exists between the analyte concentration and the TPX signal level. At analyte concentrations above the assay dynamic range a decrease in signal level known as the “hook” is seen (Figure 7).

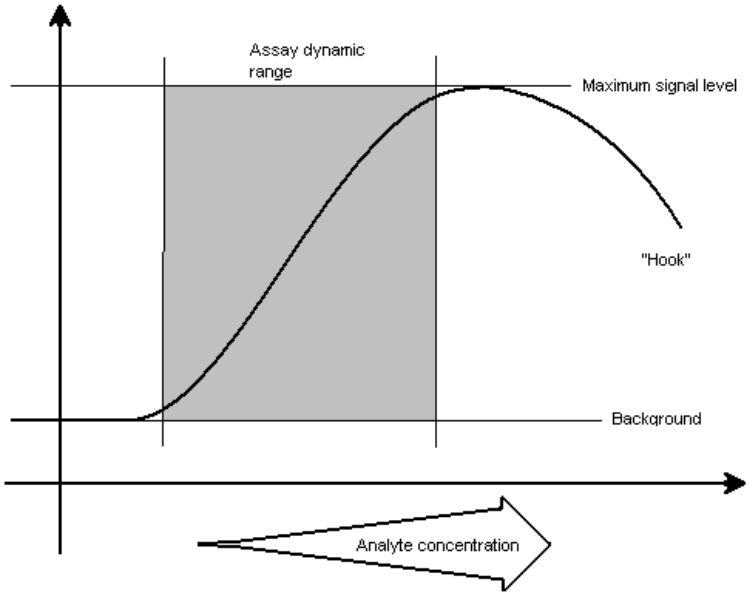


FIGURE 7. Two-photon excited fluorescence detection (TPX) immunometric assay kinetics. A direct correlation between the analyte concentration and the TPX fluorescence signal level only exists within the assay dynamic range. An increase in the analyte concentration above the assay dynamic range may result in a decrease in the signal level ("hook").

The TPX assay has so far been applied for detection of biomolecules such as C-reactive protein, oligonucleotides, and viral respiratory pathogens (Koskinen, et al., 2004; Koskinen, et al., 2007). The latest generations of the TPX instrumentation feature fully automated microtiter plate readers with point-of-care compatibility and random-access capacity. Current readers are compatible with standard 96- and 384-well microtiter plate formats.

# 3. AIMS OF THE STUDY

The purpose of the present study was to develop, evaluate, and introduce a novel phenotypic method based on the TPX technology for direct screening of MRSA colonization samples by online immunometric monitoring of bacterial growth under selective antimicrobial pressure.

The specific aims were:

1. To set up a reliable TPX MRSA assay procedure by delineating e.g., appropriate species-specific immunoreagents, selective antimicrobial agents, proper sample dilutions, and other adequate test conditions (**I, II**)

2. To verify that the TPX assay principle is valid to identify MRSA from pure cultures as well as from artificial comingled samples, and to define the diagnostic criteria for bacterial growth typical of MRSA in the TPX test conditions **(I, II)**

3. To evaluate the applicability of the TPX MRSA assay for the detection of MRSA in a large epidemiologically variable population **(III)**

4. To assess the implementation of the TPX MRSA assay for direct screening of MRSA colonization samples from various body sites **(IV)**

# 4. MATERIALS AND METHODS

## 4.1. Materials (I-IV, pre-thesis)

### 4.1.1. Bacterial isolates (I-III, pre-thesis) and colonization samples (IV)

Bacterial isolates that were examined in Studies I-IV and in the pre-thesis experiments are shown in Table 7.

**Table 7**. Bacterial isolates examined in different studies

| Study | Species | Number of isolates | Source |
|---|---|---|---|
| Pre-thesis | *Streptococcus pyogenes* | **1** | Defined strain[a] |
| **I** | MSSA[b] | 4 | Reference strains |
| | MRSA[c] | 3 | Reference strains |
| | CoNS[d] | 7 | Reference strains |
| | Total | **14** | |
| **II** | MSSA | 6 | Reference strains |
| | MRSA | 5 | Reference strains |
| | MRSA | 15 | Epidemic isolates |
| | CoNS | 7 | Reference strains |
| | Other | 1 | Reference strain |
| | Total | **34** | |
| **III** | MSSA | 6 | Reference strains |
| | MSSA | 38 | Clinical isolates |
| | MRSA | 5 | Reference strains |
| | MRSA | 243 | Epidemic isolates |
| | MRSA | 138 | Sporadic isolates |
| | CoNS | 7 | Reference strains |
| | CoNS | 43 | Clinical isolates |
| | Other | 7 | Reference strains |
| | Total | **487** | |
| **IV** | 125 colonization samples (nose, throat, groin/axillae, perineum, ulcers, catheters) collected from 14 known MRSA carriers and 16 healthy controls | | |

[a]Defined strain, group A *Streptococcus* (*Streptococcus pyogenes*) strain A200
[b] MSSA, methicillin-susceptible *Staphylococcus aureus*
[c] MRSA, methicillin-resistant *S.aureus*
[d] CoNS, coagulase-negative *Staphylococcus*

**Pre-thesis experiments.** A single defined streptococcal strain was used in these early unpublished experiments. This group A *Streptococcus* (*Streptococcus pyogenes*) strain A200 is an erythromycin-resistant clinical skin isolate (Seppälä, et al., 1998).

**Study I.** The bacterial strains were mainly purchased from the American Type Culture Collection (ATCC). These included 4 MSSA reference strains (ATCC 29213, ATCC 25923, ATCC 12600, and ATCC 43387), 1 MRSA reference strain (ATCC 43300) and 7 CoNS reference strains (*S. epidermidis* ATCC 35983, ATCC 35984, ATCC 14990, and ATCC 12228; *S. hominis* ATCC 700236 and ATCC 27844; *S. schleiferi* subsp. *coagulans* ATCC 49545). In addition, 2 previously described MRSA strains (R1 and 27r) (Tomasz, et al., 1991) were obtained from the strain collection of the National Institute for Health and Welfare (THL), Helsinki, Finland.

**Study II.** Four reference strains were purchased from the German Collection of Microorganisms and Cell Cultures (MSSA DSM 346, MSSA DSM 683, MRSA DSM 11729, and MRSA DSM 46320). In addition, the 14 strains examined in Study I were included. *Enterococcus faecalis* ATCC 29212 was used as a non-staphylococcal control strain.

Fifteen defined epidemic MRSA strains from the National Infectious Diseases Register Strain Collection (THL-NIDR, National Institute for Health and Welfare, Turku, Finland) were also included. The THL-NIDR identification numbers of the epidemic strains with corresponding collection number in the European collection of epidemic MRSA strains (HARMONY) shown in brackets were (Cookson, et al., 2007): 54511 (E6), 54518 (E7), 61608, 61974 (E1), 62158, 62176 (E10), 62396 (E2), 75626, 98442 (E19), 98462, 37481, 98541 (E24), 98819, 99524, and 99802.

**Study III.** All of the staphylococcal reference strains used in Studies I-II were included. In addition to *Enterococcus faecalis* ATCC 29212, 6 additional non-staphylococcal control strains were purchased from the ATCC collection. These were *Klebsiella pneumoniae* ATCC 27736, *Streptococcus pyogenes* ATCC 10389, *Escherichia coli* ATCC 35218, *Streptococcus pneumoniae* ATCC 49619, *Pseudomonas aeruginosa* ATCC 27853, and *Moraxella catarrhalis* ATCC 25238.

All Finnish microbiology laboratories around the country are required to send their MRSA isolates to the Staphylococcus National Reference Laboratory (SNRL) at the THL, Helsinki, for confirmation and further typing (Kerttula, et al., 2007). The main genotyping method between 1991 and 2008 was PFGE. The banding patterns were analyzed as described earlier (Vainio, et al., 2008). Until 2007, a strain was defined as epidemic if the same PFGE type was isolated from 2 or more persons. Since 2007, a strain was defined as epidemic if it was isolated from 5 or more persons.

A total of 243 representative isolates of 43 different epidemic MRSA strains (FIN 1-5 and FIN 7-44) were chosen from the SNRL collection. The characteristics of these Finnish epidemic MRSA strains have been previously described in detail (Vainio, et al., 2008). The strains represented 51 *Spa* types and 14 CCs defined by MLST (MLST-CCs 1, 5, 8, 9, 12, 22, 30, 45, 59, 72, 80, 88, 152, and 228), most of which are known to be internationally widespread. As a rule, 6 representatives of each epidemic PFGE type were selected for the study with a few exceptions (Table 8). These isolates represented practically all different epidemic MRSA types recognized in Finland between 1991 and 2009. In addition, 138 random sporadic MRSA isolates were included.

**Table 8.** MRSA[a] strains obtained from the SNRL[b] collection (Study III)

| Description | Source or strain no. |
|---|---|
| 243 epidemic isolates types FIN 1-5 and FIN 7-44 | 1 representative of FIN[c]-8 and FIN-31<br>2 representatives of FIN-23<br>5 representatives of FIN-1, FIN-20 and FIN-29<br>8 representatives of FIN-5<br>6 representatives of the other 36 PFGE types |
| 138 sporadic isolates | |

[a] MRSA, methicillin-resistant *Staphylococcus aureus*

[b] SNRL, Staphylococcus National Reference Laboratory, National Institute for Health and Welfare (THL), Finland

[c] FIN, a Finnish epidemic MRSA type as defined by pulsed field gel electrophoresis (PFGE)

The main body of the clinical non-MRSA strains consisted of 43 CoNS and 38 MSSA isolates from clinical nasopharyngeal samples of patients treated at the Turku University Central Hospital, Turku, Finland, during 2005-2009.

**Study IV.** Colonization samples of 14 previously known MRSA carriers and 16 healthy controls consisting of voluntary laboratory employees were included. All of the MRSA carriers were residents at a local specialized nursing home. The MRSA isolates had been typed by PFGE and *Spa* methods at the first encounter (Table 9).

A total of 125 MRSA colonization samples were collected. Of these samples, 61 were from previously known MRSA carriers and 64 were from healthy controls. In each case, the nose, throat, groin/axillae (treated as a single site) and perineum were

swabbed with additional samples from the patients presenting with chronic wounds or catheters. Each site was swabbed twice at the same time to produce samples for the testing by the standard enrichment culture (Swab 1) and by the TPX assay (Swab 2).

**Table 9.** Characteristics of MRSA[a] isolated from the previously known carriers

| Patient Id | *Spa*[b] | PFGE[c] |
|---|---|---|
| 1 | t008 | FIN-7[d] |
| 2 | t040 | FIN-10 |
| 3 | t040 | FIN-10 |
| 4 | t172 | FIN-4//FIN27 |
| 5 | t172 | FIN-4/FIN-27 |
| 6 | t172 | FIN-4/FIN-27 |
| 7 | t172 | FIN-4/FIN-27 |
| 8 | t037 | Sporadic[e] |
| 9 | t362 | FIN-10 |
| 10 | t040 | FIN-10 |
| 11 | ND[f] | FIN-12 |
| 12 | t040 | FIN-10 |
| 13 | t040 | FIN-10 |
| 14 | t172 | FIN-4/FIN-27 |

[a] MRSA, methicillin-resistant *Staphylococcus aureus*

[b] *Spa*, a typing method based on sequencing of the *S. aureus* protein A-gene

[c] PFGE, typing by pulsed field gel electrophoresis

[d] FIN-7, a Finnish epidemic MRSA type as defined by PFGE

[e] Sporadic, the same PFGE type isolated from only one person

[f] ND, not determined

### 4.1.2. Media and buffer solutions (I-IV, pre-thesis)

**Assay buffer (I, II, pre-thesis).** The ArcDia assay buffer (pH 8.0) was prepared into Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol, 50 mM) buffered saline (NaCl 150 mM). The buffer also contained NaN3 (10 mM), bovine serum albumin (0.5%) (BSA, fraction V; Sigma-Aldrich, Steinheim, Germany) and Tween 20 (0.01%; Sigma-Aldrich, St. Louis, MO, USA).

**Dry-chemistry buffer (I-IV).** The dry-chemistry assay buffer contained 10 mM Tris (ULTROR® Grade; Calbiochem, La Jolla, CA, USA), 50 mM NaCl (Riedel-de Haën, Seelze, Germany), 0.5% BSA, and 5% D-sorbitol (Fluka, Buchs, Switzerland), pH 7.9.

**TSB-PEG (I-IV, pre-thesis).** Autoclaved tryptic soy broth (TSB, CM0129; Oxoid, Basingstoke, UK) was supplemented with polyethylene glycol (PEG) 6000. Unless otherwise stated, the final concentration of PEG was 35 mg/ml. PEG is known to facilitate the formation of immunocomplexes (Salonen & Vaheri, 1981).

**Iso-Sensitest-PEG (I).** Semi-synthetic Iso-Sensitest broth was purchased from Oxoid (CM0473). The broth was supplemented with 3.5% PEG and then autoclaved.

All solutions were filtered through a 0.2 µm membrane before use.

### 4.1.3. Tracer (I-IV, pre-thesis)

The same 2-photon excitable fluorescent dye was used in all studies. The fluorescent dye dipyrrylmethene-BF2 530 (BF530) was obtained from Arctic Diagnostics Ltd., Turku, Finland. The tracer antibodies were labeled with the dye using a previously described standard procedure (Meltola, et al., 2005). In brief, the fluorescent dye was dissolved into dry N,N-dimethylformamide (DMF) and then mixed with an antibody solution in a 15-fold excess. After a 3-h incubation antibody-dye conjugates formed in the solution were separated from low molecular weight substances through a gel filtration column. Finally, the substitution degree (ratio of fluorescent molecules per antibody) was determined photometrically. Labeled antibodies were stored in the dark at +4°C. The antibodies used for preparation of the tracers in different studies as well as the corresponding substitution degrees are listed in Table 10.

**Table 10.** Antibodies used in preparation of immunoreagents in different studies

| Study | Cat. n:o | Source | Target | Description | BF530/Ab[a] |
|---|---|---|---|---|---|
| Pre-thesis | PAB7137P | Maine Biotech, ME, USA | Group A *Streptococcus* | Polyclonal goat IgG | 2.6 |
| I | M310127 | Fitzgerald, Concord, MA, USA | *Staphylococcus aureus* | Monoclonal mouse IgG3 | 5.6 |
| | ab20920 | Abcam, Cambridge, UK | *S. aureus* | Polyclonal rabbit IgG | 1.8 |
| II | BM3066X | Acris, Hiddenhausen, Germany | *S. aureus* | Monoclonal mouse IgG3 | 2.5 |
| | AM01227PU-N | Acris, Hiddenhausen, Germany | *S. aureus* | Monoclonal mouse IgG3 | 1.9 |
| | ab20920 | Abcam, Cambridge, UK | *S. aureus* | Polyclonal rabbit IgG | 1.8 |
| III | BM3066X | Acris, Hiddenhausen, Germany | *S. aureus* | Monoclonal mouse IgG3 | 2.9 |
| | AM01227PU-N | Acris, Hiddenhausen, Germany | *S. aureus* | Monoclonal mouse IgG3 | 4.9 |
| IV | BM3066X | Acris, Hiddenhausen, Germany | *S. aureus* | Monoclonal mouse IgG3 | 2.2 |

[a] BF530/Ab, degree of labeling. Average number of fluorescent BF530 molecules per tracer antibody

### 4.1.4. Coated microparticles (I-IV, pre-thesis)

Carboxyl-modified polystyrene microparticles for the early studies were obtained from Bangs laboratories, and later from Seradyn (Table 11). In each study the microparticles were passively coated with the same antibody used to prepare the tracer. A previously described 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) fixation method was followed (Waris, et al., 2002; Meltola, et al., 2005).

**Table 11.** Microparticles utilized in different studies

| Study | Cat. N:o | Source | Diameter |
|---|---|---|---|
| Pre-thesis | PC05N | Bangs laboratories, Fisher, IN, USA | 3.2 µm |
| I | PC05N | Bangs laboratories, Fisher, IN, USA | 3.2 µm |
| II | 7300-3420 | Seradyn, IN, USA | 3.0 µm |
| III | 7300-3420 | Seradyn, IN, USA | 3.0 µm |
| IV | 7300-3305 | Seradyn, IN, USA | 2.0 µm |

### 4.1.5. Microtiter plates and plate sealing films (I-IV, pre-thesis)

Microtiter plates with black walls and transparent µClear bottoms were purchased from Greiner Bio-One (Frickenhousen, Germany). A 384-well format (20 µl/well, cat. n:o 788096) was used in Study I and in the pre-thesis experiments while a 96-well format (half-area, 200 µl/well, cat. n:o 675096) with larger wells was later adopted. Highly gas-permeable plate sealing films from Porvair Sciences (cat. n:o 229307; Portsmouth, UK) were experimented with in Study II while conventional plastic sealing films (cat. n:o 4ti-0510; 4titude, Surrey, UK) were otherwise used.

### 4.1.6. Antimicrobials (I-IV, pre-thesis)

The antimicrobials used as selective agents for the TPX assay are listed in Table 12. In addition, during the pre-thesis trials on antimicrobial susceptibility of *Streptococcus pyogenes* A200 experiments were carried out also with penicillin and gentamycin.

**Table 12.** Antimicrobials utilized as TPX[a] assay reagents in different studies

| Study | Generic name | Cat n:o | Source | Comment |
|---|---|---|---|---|
| Pre-thesis | Erythromycin | E6376 | Sigma[b] | Serial dilutions |
| I | Oxacillin | O-1002 | Sigma | Serial dilutions |
| II | Cefoxitin | C4786 | Sigma | Single dilution |
| III | Cefoxitin | C4786 | Sigma | Single dilution |
| IV | Cefoxitin | C4786 | Sigma | Single dilution |
| | Aztreonam | 129K1255 | Fluka[c] | Single dilution |

[a] TPX, 2-photon excited fluorescence detection

[b] Sigma, Sigma-Aldrich, St. Louis, MO, USA

[c] Fluka, Buchs, Switzerland

## 4.2. Conventional microbiological analysis (I-IV, pre-thesis)

### 4.2.1. Agglutination tests (IV, pre-thesis)

**Pre-thesis experiments.** Group A *Streptococcus* was detected by a commercially available latex agglutination test Strep-A (Morwell diagnostics, Zurich, Switzerland). A bacterial stock was diluted in water and an aliquot (50 µl) was treated with Strep-A extraction reagent (400 µl). The mixture was further diluted 1:3 in series into water and

the resulting solutions were applied on Strep-A test strips. After a 10-min incubation the agglutination test results were read according to the manufacturer's instructions.

**Study IV.** Pastorex Staph Plus (Bio-Rad, Marnes-la-Coquette, France) was used for identification of *S. aureus* colonies (Weist, et al., 2006). Agglutination of the PBP 2a protein (MRSAscreen, Denka Seiken, Tokyo, Japan) was utilized in identification of pure cultured MRSA colonies. These test kits were used according to the manufacturer's instructions.

### 4.2.2. Disc diffusion method (III-IV)

Phenotypic methicillin susceptibilities for the confirmed *S. aureus* strains were determined according to the CLSI guidelines by using the standard cefoxitin and oxacillin diffusion techniques (CLSI, 2009). A colony suspension of each isolate was prepared to the density of a 0.5 McFarland standard and plated on 5% sheep blood MHA. A 1 µg oxacillin disc and a 30 µg cefoxitin disc were added onto the plate. The inhibition zone diameters were measured manually after 24 h of incubation at 37°C. The oxacillin disk was read using transmitted light according to the CLSI recommendations. *S. aureus* ATCC 25923 was used for quality control. The methicillin resistance and susceptibility breakpoints currently recommended by the CLSI for a 30 µg cefoxitin disk are 21 mm and 22 mm, respectively. The recommended breakpoints for a 1 µg oxacillin disc are $\geq$ 13 mm for susceptible, 11-12 mm for intermediate, and $\leq$ 10 mm for resistant strains. Cefoxitin test results are considered to be easier to interpret and more sensitive for the detection of MRSA (CLSI, 2009).

### 4.2.3. E-test method (I-IV, pre-thesis)

The MICs were determined by an E-test (Biodisk AB, Solna, Sweden) method according to the manufacturer's instructions. A 0.5 McFarland colony suspension was prepared and plated on 5% sheep blood MHA with an E-test strip applied on the plate surface. The plates were incubated at 37°C for 24 h in air. The MIC value was read at the point of the intersection between the visible bacterial growth zone and the Etest strip. *S. aureus* ATCC 29213 was used as a control.

### 4.2.4. Enrichment culture (IV)

Swabs from different colonization sites for the enrichment culture were collected with Copan Liquid Amies Elution Swab (ESwab) collection and preservation system (Copan Italia, Brescia, Italy). The ESwab system contains a flocked swab and 1 ml of modified liquid Amies medium, into which the sample was eluted at arrival in the laboratory. About 50 µl of the sample solution was used for culture, which included an individual enrichment in BHI containing selective concentrations of NaCl (6%),

colistin (1.32 x $10^5$ units/L), nystatin (2.2 x $10^5$ units/L), and cefoxitin (4 mg/L) (Pasanen, et al., 2009). After an overnight enrichment, 10 µl of the broth was cultured on BBL CHROMagar MRSA II (CHROMagar Microbiology, Paris, France) plate. Mauve colonies suggestive of *S. aureus* were further identified by routine methods including latex agglutination and determination of the oxacillin MIC. If the oxacillin MIC was ≤ 64 mg/L, the presence of the *mecA* gene and correct species identification were confirmed by means of the GeneXpert MRSA Nasal test (Cepheid, Sunnyvale, CA, USA), which targets the *SCCmec-orfX* junction (the *mecA* gene and adjacent *S. aureus*-specific sequences) and the *Spa* gene. A staphylococcal isolate was confirmed as MRSA if both *Spa* and *mecA* were positive by GeneXpert.

### 4.2.5. Plate count method (I-III)

A series of 10-fold dilutions were made in physiological saline. The saline dilutions were dispensed onto blood agar plates and the plates were incubated in air at +37°C overnight. The amount of viable bacteria in the original stock was calculated the next day based on the amount of visible colonies formed on the plate surfaces.

## 4.3. TPX MRSA assay (I-IV, pre-thesis)

### 4.3.1. TPX assay principle

The separation-free TPX immunometric assay principle can be applied to detection and antimicrobial susceptibility testing of specific bacteria within nonsterile specimens. In this application the same species-specific antibody is used both as the tracer and as the capture antibody. The fluorescent molecules are concentrated on microparticle surfaces via soluble bacterial antigens, cell wall fragments and whole viable cells (Figure 8). A selective antimicrobial agent is added to the mixture resulting in inhibition of growth and lysis of susceptible bacteria. Intensity of 2-photon excited fluorescence provides a quantitative measure of specific antigen concentration, which can be monitored online from a turbid and strongly scattering reaction mixture without affecting living bacteria within. Resistant concomitant microbes present in the sample are able to grow but are not recognized by the species-specific assay reagents. Thus, a rapid increase in the fluorescence signal level indicates the presence of a resistant strain of the specific bacterium.

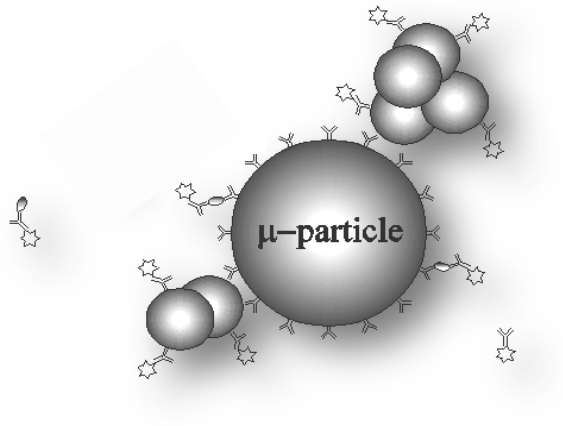


FIGURE 8. The principle of the 2-photon excited fluorescence detection (TPX) immunometric assay for detection and antimicrobial susceptibility testing of bacteria. Fluorescent molecules (stars) are concentrated on microparticle surfaces via soluble antigens (ovals) and intact bacterial cells (circles).

### 4.3.2. TPX plate reader (I-IV, pre-thesis)

An automated ArcDia TPX Plate reader PR-10 (Arctic Diagnostics Ltd.) was used in all studies. The instrument is controlled by a labtop computer running custom made software. Multiple plate formats can be used, including the common 96- and 384-formats. In these studies the reader was programmed to measure fluorescence from the assay wells for 1 to 3 min per well in a fixed pre-set order. The measurement cycle was repeated multiple times to allow kinetic measurements. During long cyclical measurements the plate inside the reader was automatically shaken by the reader mechanics for a few sec at regular 6 to 10 min intervals to prevent the settling of microparticles. The temperature of the plate inside the operating reader was passively held at approximately 32°C. The reader is shown in Figure 9.

FIGURE 9. Two-photon excited fluorescence detection (TPX) plate reader PR-10

### 4.3.3. Assay optimization (I-IV, pre-thesis)

The assay was optimized with respect to PEG, tracer and microparticle concentrations. The optimization was done by cross-testing the reagents in varying concentrations, and it was remade each time a new set of reagents was adopted. A higher tracer concentration provided a higher absolute signal but also increased the background fluorescence. A concentration that provided the highest signal-to-background ratio (SBR) was considered optimal. Theoretically, a lower microparticle concentration improves the assay sensitivity, but it also reduces the reliability of the fluorescence data analysis; thus, a longer measurement time is required. A microparticle concentration that allows approximately 100 microparticles to be found during a given measurement time was considered optimal.

### 4.3.4. Preparation of samples (I-IV, pre-thesis)

**Pre-thesis experiments.** The TSB growth medium was inoculated with a few group A *Streptococcus* A200 colonies from a blood agar plate, and the medium was then incubated overnight at +33°C to provide a bacterial stock. To prepare samples for the assay sensitivity testing the bacterial stock was subjected to nitrous acid extraction (Prolex modified nitrous acid reagents; Pro-Lab diagnostics, Richmond Hill, Ontario, Canada). 43 µl of the stock was mixed with 300 µl of both of the extraction reagents 1 and 2. After a 20-min incubation 1500 µl of the neutralizing reagent 3 was added to the mixture. The extracted solution was diluted 1:3 in series into the assay buffer containing 4% PEG. Plain assay buffer was used as a negative control.

To provide samples for testing by the erythromycin susceptibility assay the stock was diluted once 1:2000 into the TSB growth medium. No extraction step was included. PEG was not added and plain TSB was used as a negative control. Corresponding procedures were applied to penicillin and gentamicin susceptibility testing assays.

**Study I.** To study the effects of competing growth 2 bacterial stocks were prepared, one of *S. aureus* ATCC 29213 and the other of *S. epidermidis* ATCC 35983. Twenty colonies from a blood agar plate (24 h incubation at +37°C) were inoculated into the TSB-PEG medium. Both stocks were diluted in series into TSB-PEG and mixed in varying degrees. The amount of bacteria in a stock was determined by the standard plate count method. Plain TSB-PEG was used as a negative control.

The effects of the anti-*S. aureus* immunoreagents on *S. aureus* growth were studied by conventional methods alone. A bacterial stock containing *S. aureus* ATCC 29213 cells was prepared into TSB-PEG and incubated at +37°C with and without the immunoreagents (2 nM tracer, 2.5 x $10^6$ microparticles/ml). The bacterial counts were determined from both solutions by the standard plate count method at 0, 2, 3, and 6 h.

Fourteen staphylococcal reference strains were tested to determine detection limits, and to evaluate the oxacillin susceptibility test method. The isolates were stored frozen and cultured overnight at +37°C on blood agar plates before testing. Five colonies were inoculated into 1 ml TSB-PEG and incubated for another 4 h at +37°C. The suspension was then diluted 1:4 in series into TSB-PEG. Plain TSB-PEG was used as a negative control. The initial amounts of bacteria in the samples were determined by the standard plate count method.

**Study II.** The isolates were cultured in air at +37°C on blood agar plates overnight before testing. After the incubation 2 colonies were inoculated into TSB-PEG (1 ml). Low density samples were prepared by diluting the sample 1:1000 into TSB-PEG. Samples for the assay performance testing were prepared by diluting the stock 1:4 in series. Plain medium was used as a negative control.

To prepare samples for the lysozyme treatment experiment, 5 µl of the lysozyme solution (10 mg/L of sterile water) was added into a sample (*S. aureus* ATCC 12600; 300 µl) and the resulting mixture was incubated for 15 min at +37°C before testing. The amount of bacteria in a stock was determined by the standard plate count method.

**Study III.** Two colonies from a pure culture were inoculated into TSB-PEG (1 ml) and the solution was further diluted 1:1000 into TSB-PEG to provide a low-density sample for testing by the TPX MRSA assay.

**Study IV**. Sterile nylon flocked swabs (microRheologics 502CS01; Brescia, Italy) were used to collect clinical samples for testing by the TPX MRSA assay. After the sampling process the swabs were broken off and placed in a 5 ml tube to which TSB-PEG was added to provide a sample suspension. 600 µl of TSB-PEG was used with the healthy controls and 1000 µl with the MRSA-positive patients. All samples from the healthy controls and a majority of the samples from the patients were analyzed the same day within a few hours of sampling. In a few cases the samples were stored before the analysis at +4°C for up to 48 h.

### 4.3.5. Preparation of dry-chemistry plates (I-IV)

Dry-chemistry plates were used to allow a simplified assay protocol and a longer shelf-life for the reagents (Koskinen, et al., 2005). An assay reagent cocktail containing 4 times the intended final concentrations of the coated microparticles, the tracer and the antimicrobial agents was prepared in a dry-chemistry buffer. The cocktail was dispensed into the wells of a microtiter plate (25 µl/well), which was then evaporated to dryness during an overnight incubation inside a desiccator over silica gel. Finally, the plate was sealed with a gas-impermeable plastic film and stored for up to a month before use.

### 4.3.6. Assay procedure (I-IV, pre-thesis)

**Pre-thesis experiments.** A wet-chemistry approach was applied. In the assay sensitivity experiment, an assay reagent cocktail containing the tracer and the microparticles was prepared into the assay buffer (+4% PEG). For the erythromycin susceptibility assay, 5 different reagent cocktails were prepared into TSB, each containing a different erythromycin concentration (0, 0.01 x MIC, 0.1 x MIC, 1 x MIC, and 10 x MIC) in addition to the immunoreagents. The reagent cocktails were mixed 1:1 with the samples, and 20 µl of each mixture was dispensed into sample wells of a 384-well plate. After 1 h of incubation at room temperature the plate was measured. For the assay sensitivity test the plate was measured once (60 s/well). For the erythromycin susceptibility testing the plate was measured repeatedly for a period of 18 h under growth sustaining conditions. Similar steps were applied to penicillin and gentamicin susceptibility testing assays, with the final antimicrobial concentrations around the pre-determined MIC values of these agents for *S. pyogenes*.

**Study I.** 384-well dry-chemistry plates were employed to allow a simplified assay protocol. The 20 µl samples were dispensed into the wells containing the immunoreagents and the antimicrobials in a dry state. The final oxacillin concentrations were 0, 0.25, 1, 4, 16, and 64 mg/L. The plate was then sealed and incubated for 30 min to allow dissolution of the reagents. The plate was measured repeatedly for a period of 10 h under growth sustaining conditions.

**Study II.** 96-well dry-chemistry plates with 100 µl sample volumes were employed. The single cefoxitin concentration was 4 mg/L. After inoculation of all samples the plate was sealed and incubated at room temperature for 30 min. Fluorescence signal levels were monitored for up to 20 h under growth sustaining conditions by repeated measurements for 3 min per a sample well.

**Study III.** 96-well dry-chemistry plates containing the assay reagents and cefoxitin (4 mg/L) were employed. After a 30-min incubation the plate was measured repeatedly for 2 min per well for a period of 19 h under growth sustaining conditions.

**Study IV.** In addition to each sample 1 dilution of it was prepared into TSB-PEG. The dilution factor was 100 for the controls 1 to 6; 10 for the controls 7 to 16 and the patients 1 to 4; and 20 for the patients 5 to 14. The sample and the dilution (100 µl each) were dispensed into 2 separate wells of a dry-chemistry 96-well plate. All wells contained cefoxitin (4 mg/L) and aztreonam (20 mg/L). After dispensing all samples and dilutions the plate was re-sealed and incubated at room temperature for about 30 min. Fluorescence was then measured repeatedly for 2 min per assay well for a period of 13 h in growth sustaining conditions.

After the test run the plate sealing film was removed and 20 µl from each sample well was inoculated on BBL CHROMagar MRSA II agar (BD Diagnostics, Sparks, Md, USA). The plates were incubated at +37°C in air for 24 h and then examined for colonies morphologically suggestive of *S. aureus*. Any such colonies were collected for confirmation by PCR (THL, Helsinki). An isolate was considered MRSA if both *mecA* and *nuc* genes were detected.

### 4.3.7. Fluorescence data analysis (II-IV)

Hundreds of short distinct bursts of 2-photon excited fluorescence (particle signals) were recorded during a TPX measurement lasting a couple of minutes. Each burst represents 2-photon excited fluorescence from an individual microparticle with the intensity proportional to the amount of the tracer molecules bound on the surface. The R-environment (http://www.r-project.org/), with a robust mean signal estimation algorithm for statistical computing was used to reduce these particle signals to a single TPX fluorescence signal value (Glotsos, et al., 2006). The more bursts that are

available for the statistical analysis, the more reproducible the fluorescence signal value provided by the algorithm is. A TPX fluorescence signal value was discarded from any further analysis if less than 10 particle signals were detected during a measurement. To compensate for the effects of the fluorescent components of the complex medium the particle signals were normalized to the solution fluorescence.

In kinetic assays each well was measured multiple times at regular intervals to provide time points for a fluorescence-versus-time [*F(t)*] curve. Up to 18 consecutive TPX fluorescence signal values were recorded for a single well. The data was further reduced to a single numeric value by determination of the maximal fluorescence signal increase rate [max($\Delta F/\Delta t$)]: the maximal difference between 2 consecutive fluorescence signal values divided by the time interval in between, i.e. an approximation of a tangent to the steepest part of the *F(t)* curve for an assay well. The max($\Delta F/\Delta t$) for the MRSA-negative samples + 3 x standard deviations (3SD) was considered to be a significant rate in the increase in fluorescence and indicated a positive TPX MRSA assay result. A max($\Delta F/\Delta t$) value was considered inconclusive if there were 2 consecutive or a total of 3 or more discarded fluorescence signal values.

The Mann-Whitney U-test and standard receiver operating characteristic (ROC) analysis were used to differentiate between the MRSA-positive and negative samples with respect to the max($\Delta F/\Delta t$) values. *P* values < 0.05 were considered statistically significant.

In Study IV the max($\Delta F/\Delta t$) values were independently determined for the sample and the dilution well. The higher conclusive value was subjected to ROC analysis with 1 exception: if the initial fluorescence signal value of the sample well was > 10 [× fluorescence background signal level/h], the max($\Delta F/\Delta t$) determined for the diluted well was considered. The final TPX test result was considered inconclusive if both the sample and the dilution were inconclusive. These results were excluded from the performance analysis.

### 4.3.8. Interpretation of TPX assay test results (IV)

The conventional enrichment culture and the TPX MRSA assay were performed on 2 different swabs. As a result it is possible that MRSA was sometimes present in only 1 of the 2 swabs. In cases where the results of the enrichment culture (Swab 1) and the TPX assay (Swab 2) were inconsistent, the result of the recovered TPX reaction mixture culture was used to confirm either one. The TPX assay of a colonization site was defined as false-positive, if the result was positive but the enrichment culture was negative and MRSA was not isolated from the TPX reaction mixture. The TPX assay of a colonization site was defined as false-negative, if the result was negative but MRSA was isolated from both the enrichment culture (Swab 1) and the TPX reaction

mixture (Swab 2). The TPX assay of a site was defined as true-positive or true-negative, if the result was consistent with that obtained from the enrichment culture and/or the TPX reaction mixture culture.

A person was determined to be MRSA true-positive by the TPX technology, if at least 1 colonization site examined was MRSA true-positive by the above criteria. A person was determined to be MRSA false-positive by this technique, if at least 1 site examined was defined as positive by the TPX technique, but all sites examined were negative by the control methods. A person was determined to be MRSA true-negative by the TPX technology, if all colonization sites examined were negative by the TPX assay as well as by the control methods. A person was determined to be MRSA false-negative by this technique, if all sites were negative by the TPX assay but at least 1 site was defined as positive by the control methods.

# 5. RESULTS

## 5.1. Pre-thesis experiments with the TPX technique in bacteriology

### 5.1.1. Assay analytical sensitivity (pre-thesis)

To evaluate the performance of the TPX-based immunological assay in detection of group A *Streptococcus* antigens, the sensitivity of the TPX assay was compared to a commercially available slide agglutination test Strep-A. Both of these methods included an extraction step to release intracellular antigens into the medium.

Differentiation of the positive and negative Strep-A test results was hampered by the subjective nature of the visual detection. The highest dilution to produce a positive result in the Strep-A test was determined to be 1:810 with respect to the original stock.

Standard curves determined by the TPX assay are shown in Figure 10. Even though suggestive in nature, the experiment showed that the highest dilution that could be differentiated from the negative control by 3SD is about 2 orders of magnitude higher in the TPX assay than the highest dilution visually recognizable as positive by the commercial Strep-A test.

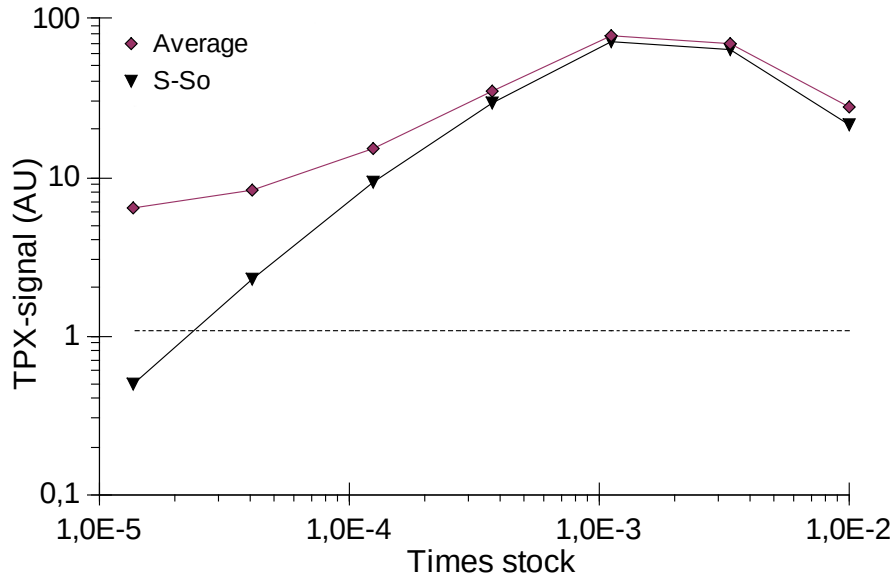


FIGURE 10. Various dilutions of a bacterial stock containing group A *Streptococcus* A200 were measured by the 2-photon excited fluorescence detection (TPX) assay. The absolute TPX signal level (in arbitrary units) is shown as a function of the dilution factor. Six replicate wells were used and the average signal level is shown. The curve

below is background subtracted (S-So). A dotted line marks the level of the negative control + 3 x standard deviation (3SD) (3 x So Std. dev). The intercept of the lower curve with the dotted line indicates the lowest detectable dilution, which was approximately 1/80000. Reagents were prepared with polyclonal anti-group A *Streptococcus* PAB7137P goat IgG antibodies.

### 5.1.2. TPX-based antimicrobial susceptibility testing (pre-thesis)

The erythromycin MIC for the *Streptococcus pyogenes* A200 strain was determined by the conventional methods to be 8 mg/L. The fluorescence signal level of 1/2000 diluted bacterial stock in the presence of varying erythromycin concentrations was monitored online by repeated TPX measurements for a period of 18 h. The resulting growth [F(t)] curves are presented in Figure 11.

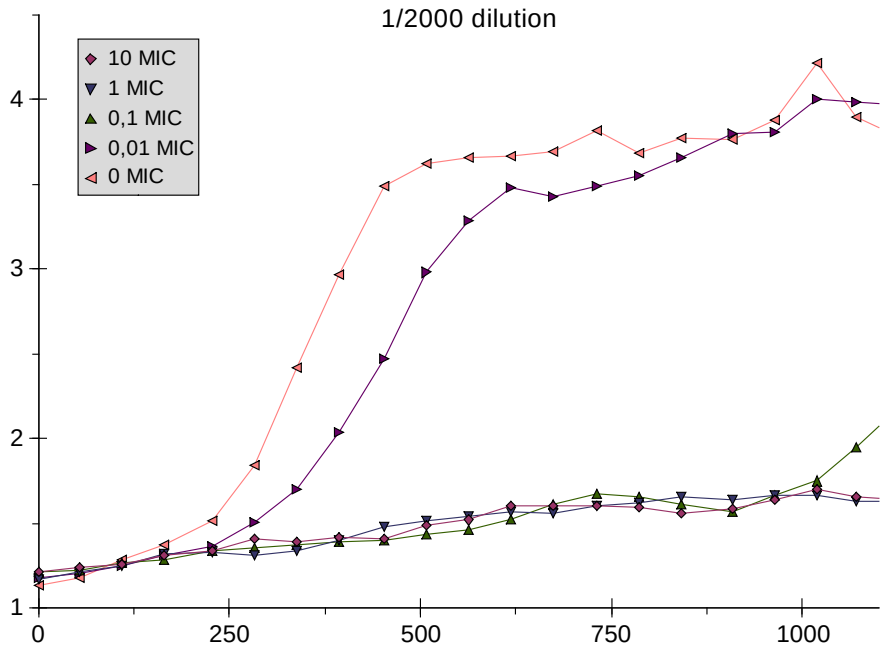


FIGURE 11. Fluorescence-versus-time [F(t)] ([AU]/[min]) curves for the 1/2000 diluted erythromycin-resistant *Streptococcus pyogenes* strain A200 solution containing varying erythromycin concentrations. Bacterial growth is completely inhibited in solutions containing 1 MIC or more of erythromycin. Growth rates are decreased in solutions containing 0.1 MIC and to a lesser degree in 0.01 MIC. The TPX signal level is related to background fluorescence level. Reagents were prepared with polyclonal anti-group A *Streptococcus* PAB7137P goat IgG antibodies.

When either penicillin or gentamicin was used as the selective antimicrobial agent, fluorescence signal progression was interfered about around 1 x MIC concentrations (data not shown).

## 5.2. Development of the TPX MRSA technology (I-II)

### 5.2.1. Evidence for validity of the TPX MRSA assay principle (I)

As a proof of the TPX MRSA assay principle growth [*F(t)*] curves were determined for 14 staphylococcal reference and well-defined strains in various oxacillin concentrations (0, 0.25, 1, 4, 16, and 64 g/L). Included were 3 MRSA, 4 MSSA and 7 CoNS strains, which were examined by the TPX technique with different sizes of inoculums.

For the 4 MSSA strains, fluorescence signal progressions were interfered around oxacillin concentrations of 1 x MIC. The fluorescence signal increase for MSSA ATCC 12600 (MIC = 0.5 g/L) was delayed at an oxacillin concentration of 1 g/L, and prevented at a concentration of 4 g/L. Similarly, the signal rises for the MSSA ATCC 43887 (MIC = 0.5 g/L), ATCC 25923 (MIC = 0.25 g/L), and ATCC 29213 (MIC = 0.25 g/L) were delayed at an oxacillin concentration of 1 g/L, and prevented at a concentration of 4 g/L. In oxacillin concentrations above 1 x MIC the fluorescence signals were found to rise linearly with respect to the size of the *S. aureus* inoculums (Figure 12).

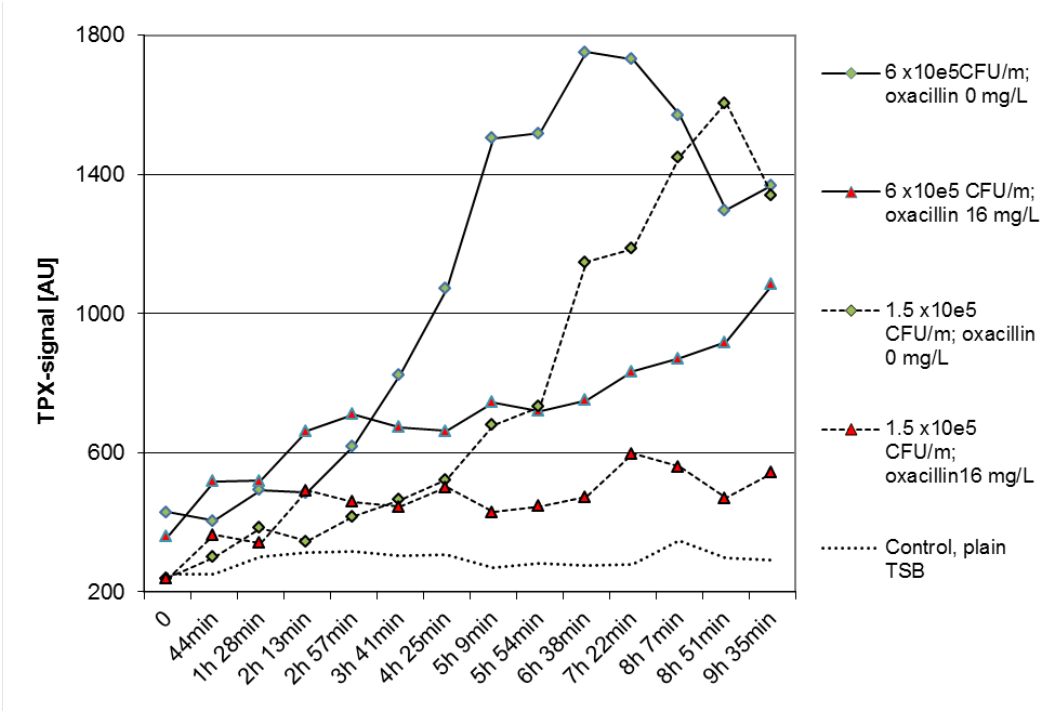


FIGURE 12. Fluorescence-versus-time [*F(t)*] curves recorded for methicillin-susceptible *Staphylococcus aureus* ATCC 12600 (oxacillin MIC = 0.5 mg/L) with various size inoculums (initial cell counts shown) and in different oxacillin concentrations.

Among the 3 MRSA strains, the data regarding the growth of an MRSA reference strain ATCC43300 (MIC = 4 mg/L) showed that the fluorescence signal rise was significantly delayed at an oxacillin concentration of 4 g/L, and prevented at a concentration of 16 g/L. The growth of the previously described MRSA strain R1 (MIC = 16 g/L) was delayed, nearly inhibited, at a concentration of 16 mg/L, and inhibited at a concentration of 64 mg/L. The previously described highly resistant MRSA strain 27r (MIC > 128 g/L) was able to grow at all oxacillin concentrations.

In examining the specificity of the polyclonal anti-*S. aureus*-antibody reagents used, only 1 (*Staphylococcus schleiferi* subsp. coagulans ATCC 49545) of the 7 CoNS strains studied exhibited fluorescence signal levels above the zero control (3SD rule). This was evidently due to cross-reactivity with the polyclonal antibody tested. Still, the fluorescence signal remained at a distinctly lower level than that of any *S. aureus* strain. Other CoNS strains showed flat line responses.

### 5.2.2. Assay optimization

The determined optimal microparticle and tracer concentrations used in all analyses are presented in Table 13. A 3.5% PEG 6000 concentration was determined optimal for the TPX assay performance. In Study I the monoclonal anti-*S. aureus* M310127 antibody was found to present substantial nonspecific binding.

**Table 13.** Optimal microparticle and tracer concentrations in different studies

| Study | Antibody | Description | Tracer [nM] | µ-particles [pcs/ml] | SBR[a] |
|---|---|---|---|---|---|
| Pre-thesis | PAB7137P | Polyclonal | 2 | 2.5x10e6 | NA[b] |
| I | M310127 | Monoclonal | NA | NA | NA[c] |
| | ab20920 | Polyclonal | 2 | 2.5x10e6 | 3 |
| II | BM3066X | Monoclonal | 2 | 5.0x10e6 | 34 |
| | AM01227PU-N | Monoclonal | 4 | 5.0x10e6 | 12 |
| | ab20920 | Polyclonal | 2 | 2.5x10e6 | 3 |
| III | BM3066X | Monoclonal | 8 | 2.5x10e6 | 15 |
| | AM01227PU-N | Monoclonal | 2 | 2.5x10e6 | 13 |
| IV | BM3066X | Monoclonal | 4 | 5.0x10e6 | 26 |

[a] SBR, signal-to-background ratio in detection of *Staphylococcus aureus ATCC 12600* whole cells
[b] NA, not applicable
[c] NA, insufficient performance for the monoclonal anti-*S. aureus* M310127 antibody

### 5.2.3. Effects of competing microbes and immunoreagents on *S. aureus* growth (I)

Samples containing various amounts of live *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35983 cells were monitored by the TPX assay under growth sustaining condition for 10 h. The growth [*F(t)*] curves measured for the various mixtures are presented in Figure 13. The study revealed that a 35-fold excess of *S. epidermidis* compared with *S. aureus* had no significant effect on the assay performance in monitoring *S. aureus* growth. However, a 350-fold excess lowered fluorescence signal levels by approximately 40%. When studied by conventional methods, the immunoreagents (microparticles and tracer) did not have any significant effect on *S. aureus* growth rate at concentrations commonly applied by the TPX assay.

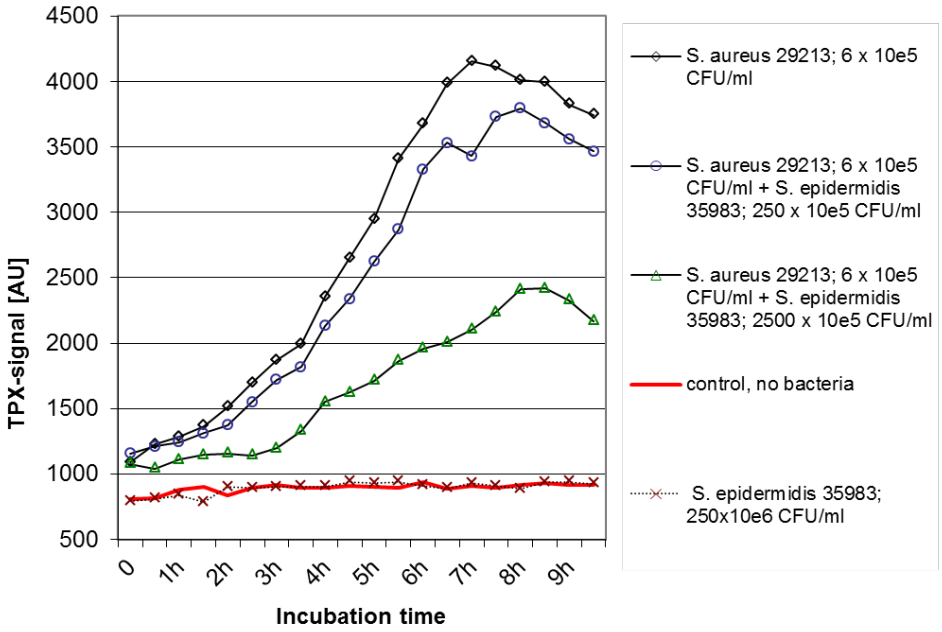


FIGURE 13. Effects of competing *Staphylococcus epidermidis* ATCC 35983 growth on *Staphylococcus aureus* ATCC 29213 growth monitored by the 2-photon excited fluorescence detection (TPX) assay. Assay reagents prepared with polyclonal anti-*S. aureus* antibody ab20920.

### 5.2.4. Detection of *S. aureus* whole cells and lysozyme-treated cells (II)

In detection of *S. aureus* whole cells immunoreagents prepared with the monoclonal anti-*S. aureus* BM3066X antibody provided the best performance, i.e. the highest SBR value (Table 13). The assay detection limit with these reagents for the MSSA ATCC 25923 whole cells was determined to be 5 x $10^4$ CFU/ml. However, a low-density sample that initially contained only 2 x $10^3$ CFU/ml of ATCC 25923 cells was detectable after 2.5 h of measurement. This would imply exponential growth with doubling times of about half an hour in the assay conditions (gas-impermeable sealing, +32°C, TSB-PEG medium). A highly gas-permeable plate had no significant effect on the assay performance.

An assay dose-response curve (a fluorescence-bacterial cell count [*F(c)*] curve) with respect to the cell count from 8 sample dilutions of MSSA ATCC 25923 whole cells is shown in Figure 14. Consistent with all reagent-limited assay methods, the dose-response curve was characterized by a flat-line response at analyte concentration below the detection limit, by a nearly linear portion within the dynamic range of the assay, and by a hook effect with excess analyte concentrations.

Approximately 30% higher absolute fluorescence signal levels were achieved with respect to the bacterial count when MSSA ATCC 12600 stock was first treated with lysozyme to release intracellular antigens (Figure 14).

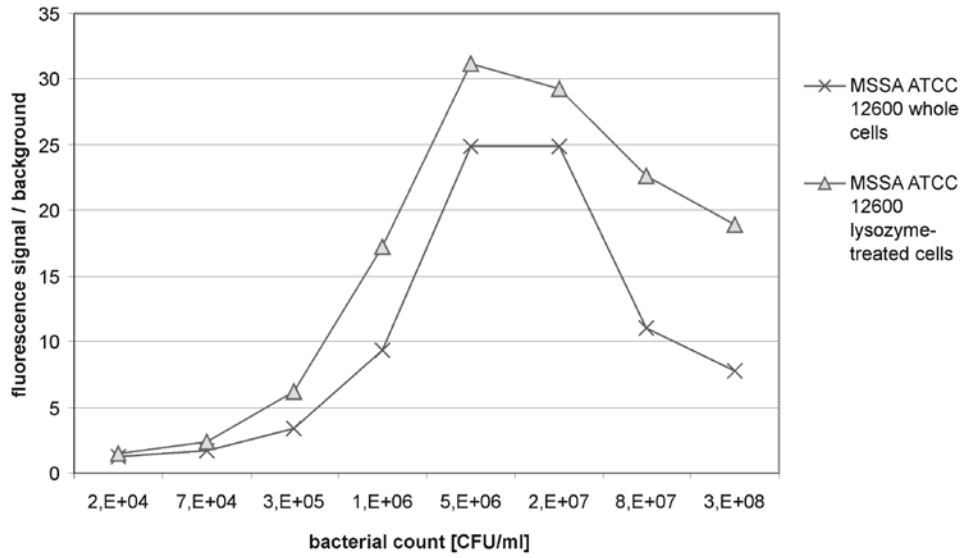


FIGURE 14. Fluorescence-bacterial cell count [*F(c)*] curves for methicillin-susceptible *Staphylococcus aureus* strain ATCC 12600 whole cells and lysozyme-treated cells with respect to the initial cell count. Lysozyme treatment disintegrates the bacteria and releases intracellular antigens in the process. Assay reagents prepared with the monoclonal anti-*S. aureus* antibody BM3066X were used (Study II).

### 5.2.5. Recognition of MRSA from samples containing also MSSA or CoNS (II)

MSSA and CoNS are frequently present in colonization samples being screened for MRSA. Hence, the performance of the TPX assay was tested with specimens containing different concentrations of MSSA or CoNS, in addition to MRSA. The results showed that 27-fold or lower excess for MSSA, or 16-fold or lower excess for CoNS had no significant effect on the assay performance.

### 5.2.6. Growth [*F(t)*] curves typical of MRSA in TPX assay conditions (II)

Max(ΔF/Δt) values were determined for 33 well-defined MRSA, MSSA and CoNS strains**.** A breakpoint max(ΔF/Δt) value of 2.5 (x fluorescence background level/h) was defined as a significant rate in the increase in fluorescence, indicating a positive TPX test result. All samples containing MRSA but none of those containing MSSA or

CoNS had max(ΔF/Δt) values above the level needed to achieve a significant difference ($P < 0.001$).

Growth typical of MRSA in the TPX conditions is illustrated by separate *F(t)* curves for 20 well-defined MRSA strains (Figure 15). These curves are all characterized by a rapid increase in the fluorescence signal levels. For comparison, growth typical of MSSA or CoNS is illustrated by separate *F(t)* curves for 6 MSSA and 7 CoNS strains (Figure 16). These curves are characterized by significantly slower responses as compared with those of MRSA.

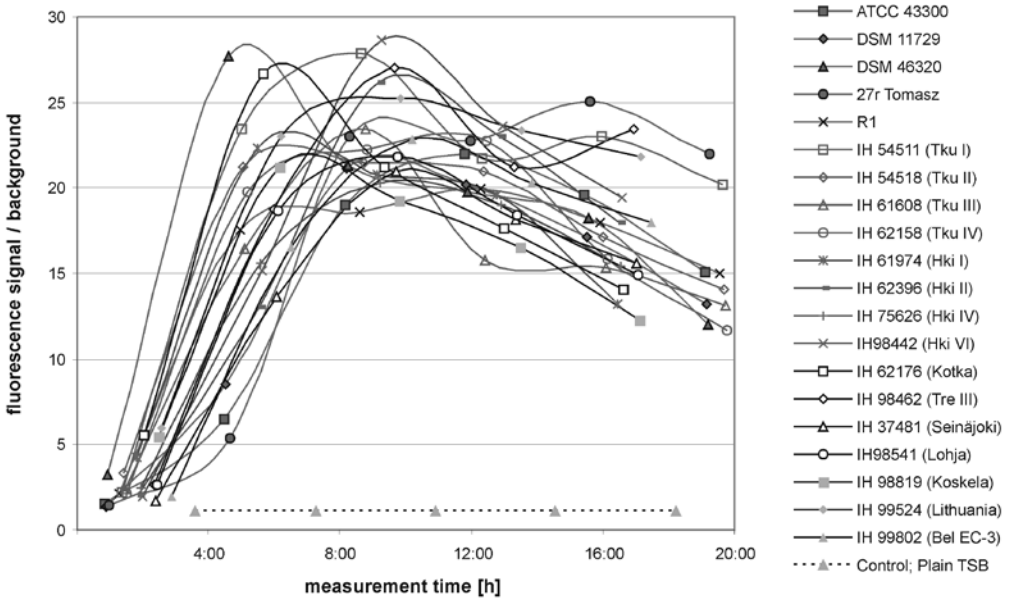


FIGURE 15. Fluorescence-versus-time [*F(t)*] curves recorded for 20 h from low-density samples with 20 defined methicillin-resistant *Staphylococcus aureus* strains. Cefoxitin was present at a concentration of 4 mg/L. Assay reagents prepared with the monoclonal anti-*S. aureus* antibody BM3966X were used (Study II)

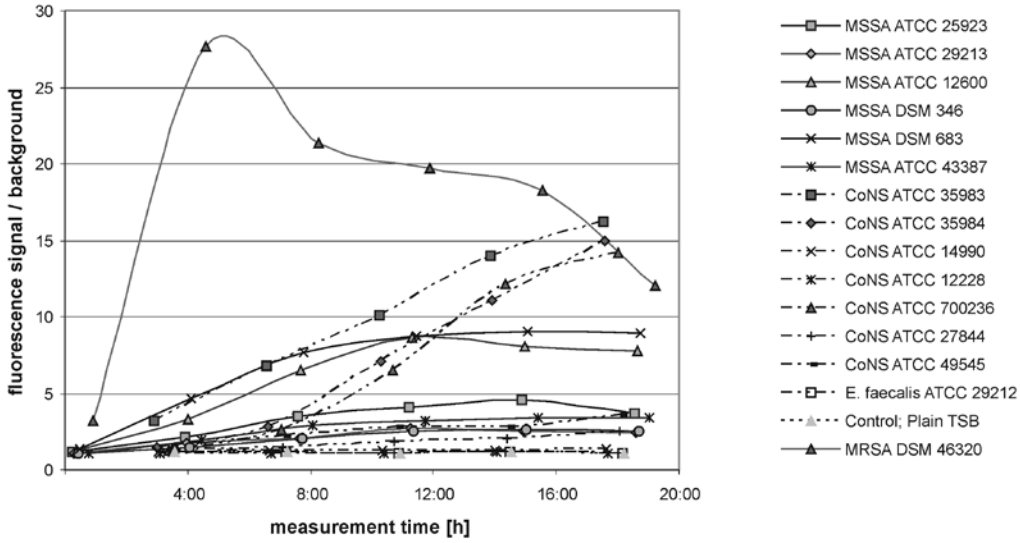


FIGURE 16. Fluorescence-versus-time [*F(t)*] curves recorded for 20 h from low-density samples with 6 methicillin-susceptible *Staphylococcus aureus* strains, 6 coagulase-negative *Staphylococcus* strains, and 1 *Enterococcus faecalis* strain as a negative control. A methicillin-resistant *S. aureus* strain was included as a positive control. Cefoxitin was present at a concentration of 4 mg/L. Assay reagents prepared with the monoclonal anti-*S. aureus* antibody BM3966X were used (Study II).

In this limited study collection, the assay exhibited 100% sensitivity and 100% specificity in detection of MRSA when reagents prepared with the monoclonal anti-*S. aureus* BM3066X antibody were used. When the reagents were prepared with the monoclonal AM01227PU-N antibody the assay was also 100% sensitive and specific, although the SBR was considerably lower. The measurement time required to confirm all MRSA true-positive samples as positive was 8 h when reagents prepared with the monoclonal BM3066X antibody were used while a longer measurement time of 12 h was required when reagents were prepared with the monoclonal AM01227PU-N. If the reagents were prepared with the polyclonal anti-*S. aureus* antibody ab20920 the assay sensitivity was only 70%. The assay capacity was up to 70 samples in a single test run.

## 5.3. Detection of MRSA from pure cultured colonies (III)

The assay tolerance to wide biological variation was evaluated by screening a large collection of 487 isolates, including 386 MRSA isolates, for MRSA. Reagents were prepared with the BM3066X antibody. Based on standard ROC analysis a breakpoint max($\Delta F/\Delta t$) value of 0.6 (x fluorescence background level/h) was determined with higher values indicating a positive test result. The test was true-positive for 97.9%

(378/386) of all MRSA isolates included. The test was true-negative for 95.5% (42/44) of the MSSA isolates and for 92% (46/50) of the CoNS isolates included. All non-staphylococcal isolates were negative by the TPX assay. Thus, the assay sensitivity was 97.9% and the specificity was 94.1% while the positive predictive value (PPV) was 98.4% and the negative predictive value (NPV) was 92.2%.

There were 8 MRSA isolates that gave false-negative screening test results. Seven of them were sporadic isolates. One had a wide cefoxitin inhibiton zone of 26 mm whereas the rest were clearly resistant by the disc diffusion method. Three of the 8 MRSA false-negative isolates as detected with the monoclonal anti-*S. aureus* antibody BM3066X showed characteristic MRSA-type growth [*F(t)*] curves when re-evaluated using alternative reagents prepared with the monoclonal anti-*S. aureus* AM01227PU-N antibody.

Two MSSA isolates and 4 CoNS isolates produced MRSA false-positive test results. Four of them were weakly positive, while 2 CoNS isolates exhibited high max($\Delta F/\Delta t$) values, being able to grow in the TPX test conditions.

An average time required before a significant max($\Delta F/\Delta$) value was recorded for a MRSA true-positive strain was 8 h, and 99% of the true-positive test results were confirmed in less than 14 h. Negative test results were confirmed after 19 h. The assay capacity was 48 samples in a test run.

## 5.4. Direct screening of colonization samples for MRSA (IV)

### 5.4.1. Conventional analysis

Of the 125 colonization samples 61 were collected from the 14 patients and 64 from the 16 controls. MRSA was detected from 37/52 enrichment cultures (Swab 1) of colonization samples of 12 patients, and from 29/48 TPX assay reagent mixture cultures (Swab 2) of 11 patients. For 2 patients, all sites (9/9) examined were negative by both of these methods, suggesting that MRSA carriage had potentially been eradicated. None of the samples collected from the controls was positive for MRSA by either conventional method.

### 5.4.2. TPX MRSA assay

An optimal max($\Delta F/\Delta t$) breakpoint value was determined to be 3.5 (x fluorescence background level/h) with higher values indicating a positive TPX MRSA test result. All 12 patients positive for MRSA by either of the conventional methods were determined to be MRSA-positive also by the TPX technology. The TPX MRSA assay was defined as false-positive for 1/14 patients and for 3/16 controls. The assay was

defined as true-negative for 1/14 patients and 13/16 controls. False-negative test results were not encountered. Thus, in identifying MRSA-positive patients, the sensitivity of the TPX assay was 100% while the specificity was 78%, the PPV was 75%, and the NPV was 100%.

The TPX assay performance was also analyzed per a colonization sample. In case of disagreement between the TPX assay and the enrichment culture, the result of the TPX reaction mixture culture was used to confirm either result. Subsequently, 31/61 colonization samples from the patients were defined as TPX MRSA true-positive while 19/61 were defined as true-negative. Further, 2/61 patient samples were defined as MRSA false-positive while 4/61 patient samples were defined as MRSA false-negative. The TPX test results were determined false-positive for 4 and true-negative for 59 samples from the controls. Inconclusive results were defined for 14 (11%) of all undiluted samples. For 5/61 patient samples and 1/64 control samples both the sample and the dilution were inconclusive rendering the final TPX test result for that colonization site inconclusive. Due to the missing results, these samples were excluded from the analysis when the TPX assay performance was determined. Subsequently, the sensitivity of the TPX assay per colonization sample was 89% and the specificity was 93%. The PPV of the assay was 84% and the NPV 95%.

A majority (71%) of the inconclusive samples were from throat, 14% were from groin/axillae and 7% were perineal samples. Of the 1:10 dilutions, 5% (3/58) and of the 1:20 dilutions, 7% (3/43) were inconclusive while none the 1:100 dilutions was inconclusive.

An average time required before a significant max($\Delta F/\Delta$) value was recorded for an MRSA true-positive sample was 4 h 35 min. The median time required for the confirmation of a true-positive TPX MRSA screening test result was 3 h and 26 min (range 1 h 20 min to 11 h 27 min). The assay capacity was 48 samples in a test run.

# 6. DISCUSSION

A wide-scale screening of patients with a high risk of MRSA carriage is one of the mainstays of a stringent MRSA control policy. Therefore, it is regrettable that all of the present-day MRSA screening methods have a number of limitations. The conventional phenotypic methods commonly used in routine diagnostic laboratories because of their easy performance and low expenses are slow, whereas the rapid real-time PCR assays are hampered by technical complexity, high price and a low-throughput capacity. A rapid culture-based non-molecular MRSA screening test, the BacLite Rapid MRSA, has been shown to detect MRSA nasal colonization within 5 h, but ciprofloxacin-susceptible MRSA strains are not identified by this test (Johnson, et al., 2006). This hinders especially the recognition of those CA-MRSA strains which are still susceptible to ciprofloxacin. The present study was commenced to develop and introduce a novel TPX-based phenotypic automatic method for direct screening of MRSA, suitable for use also in routine clinical laboratories.

## 6.1. Development of a novel TPX-based MRSA screening assay

### 6.1.1. Preliminary steps

In the Laboratory of Biophysics, University of Turku, research by means of the TPX technology has been conducted since the 1990s (Hänninen, et al., 1994). The bioaffinity detection technique is based on 2-photon excitation of fluorescence and polystyrene microspheres, which act as solid-phase reaction carriers for the immunocomplex formation (Soini, et al., 2002). Due to the separation-free detection, the technique allows online monitoring of bioaffinity reactions (Koskinen, et al., 2006). The first experiments with potential applications of the TPX methodology in the field of bacteriology were made in 2004 by MSc Joanne Vaarno and myself. Our preliminary research was focused on the genus *Streptococcus* with the initial aim to develop a highly sensitive TPX-based immunological assay to replace agglutination tests used to detect group A *Streptococcus* from throat samples. The TPX assay was shown to have a 2 orders of magnitude higher analytical sensitivity in detection of group A streptococcal antigens than the commercial StrepA agglutination test it was compared with. However, it seemed likely that the use of the TPX method as an identification assay for group A *Streptococcus* would be limited by a considerably longer incubation time of 1 h compared to the recommended incubation time of 10 min in the Strep-A test. At this early stage, also the effect of an antimicrobial agent, erythromycin, on the growth of group A *Streptococcus* was evaluated by means of the TPX technique. The first assay included a matrix of 2 sample dilutions and multiple antibiotic concentrations to allow identification and erythromycin susceptibility testing of group A *Streptococcus* in a single-step process. Gradually slower fluorescence

signal level progressions were observed with increasing concentrations of the antibiotic and flat line responses were measured above concentrations of 1 x MIC. Corresponding results were obtained when either penicillin or gentamycin was used as a selective antimicrobial agent.

Before long, this research line was discontinued because of an estimated minor clinical relevance of an additional group A *Streptococcus* test. Nevertheless, these early experiments provided valuable fundamental data. They showed that the growth of bacteria can be initiated and sustained in a TPX instrument. They also showed that it is possible to monitor the growth process by periodical TPX measurements of the amount of specific bacterial antigens within the reaction volume, without causing any significant interference with the course of growth itself. As well, the experiments showed that the concept of the TPX methodology is suitable for antimicrobial susceptibility testing of bacteria.

These preliminary experiments served as groundwork and bestowed stimulus upon the commencement of the present work. The idea was born that the TPX methodology could be applied for the phenotypic detection of MRSA directly from non-sterile clinical samples, especially from colonization samples of suspected MRSA carriers, by online immunometric monitoring of bacterial growth under selective antimicrobial pressure.

### 6.1.2. Planning and organizing

Besides being sensitive and specific, the requirements for an up-to-date assay for the screening of MRSA from clinical colonization samples are manifold. In recent years, a number of new improved methods for detection of MRSA have been presented, but none of them has been successful in meeting all of the existing clinical demands. Primarily, an optimal assay should have a high-throughput capacity, as an abundance of potential carriers must be tested for MRSA during outbreak situations. Other major requirements include e.g., short turnaround time, cost-effectiveness, possibility for automatic reading of the test results, and suitability even for point-of-care testing. The technique should be practical in a routine clinical setting without highly trained laboratory personnel.

Setting up a novel TPX-based bacteriological assay formula for MRSA diagnostics was a challenge. First of all, the suitability of the assay principle for such a procedure had to be verified. The postulation was that during incubation in a growth medium simultaneously with *S. aureus*-specific antibodies, a fluorescently labeled tracer, and a penicillinase-resistant beta-lactam antibiotic, MSSA and methicillin-susceptible CoNS are inhibited by the selective antimicrobial agent, while MRSA and methicillin-resistant CoNS are able to grow. The *S. aureus*-specific antibody then recognizes

MRSA but not CoNS antigens. Consequently, a rapid increase in the TPX fluorescence signal level indicates that living MRSA cells are present in the sample and confirms a positive MRSA screening test result. As a phenotypic method, the TPX MRSA assay was designed for screening purposes. Currently, a definite identification of methicillin resistance in any *S. aureus* strain should still be based on the detection of the presence of the *mecA* gene. In the future, this designation apparently needs revision due to the novel discovery of a divergent *mecA* homologue, *mecC*. Inclusion of tests for the *mecC* gene should be considered when new diagnostic guidelines for the detection of MRSA are outlined (García-Álvarez, et al., 2011).

Also, proper assay reagents and test conditions for a TPX-based MRSA test had to be delineated and established. The main prerequisite was the finding and validating of a species-specific antibody with an adequate capacity to detect *S. aureus* isolates. Suitable antimicrobial agents had to be defined for inhibition of the growth of methicillin-susceptible staphylococci as well as other microbes potentially present in colonization samples. A basic issue was characterization of a bacterial growth pattern typical of MRSA in the TPX study conditions. In addition, there were many other important topics to be addressed and solved. These included e.g., the assay settings, i.e. the amount of sample dilutions required, the conditions inside the TPX reader, the production of dry-chemistry plates, etc. At the beginning, the research was focused on pure cultured isolates of various reference strains and other well-characterized bacterial strains. When only pure cultures were analyzed, it had to be taken into account that many factors associated with the potential applications of the new concept to examine clinical colonization samples with various amounts and types of bacteria remained unclear, leaving those issues to be examined later. For example, the influence of host flora as competing microbes was initially unknown. Furthermore, it was not known how metabolically active bacteria just colonizing the host would be. Neither was it known, whether the inocula used to examine pure cultured isolates would suit a clinical situation at various colonization sites.

### 6.1.3. Detection of *S. aureus* by immunoreagents

An optimal antibody to be used in the TPX MRSA screening assay should be highly sensitive towards *S. aureus* but show only minimal cross-reactivity with CoNS and potential other microbes in colonization samples. A false recognition of CoNS as *S. aureus* by an unspecific antibody (Blake & Metcalfe, 2001) would be detrimental, considering the general methicillin resistance among modern nosocomial CoNS strains (Huletsky, et al., 2004). During the present study, 4 different anti-*S. aureus* antibodies were tested. First, a monoclonal anti-*S. aureus* antibody was cross-tested in various combinations with a polyclonal antibody (I). The monoclonal M310127 antibody presented an unacceptable level of nonspecific binding; thus, the polyclonal anti-*S. aureus* Ab20920 antibody was chosen for further studies both in capture and tracer

roles. It was assumed that a polyclonal antibody with multiple target antigens would react with a wider array of different *S. aureus* strains than a monoclonal antibody would.

Later on, however, the polyclonal anti-*S. aureus* antibody performed poorly exhibiting a low SBR and a large strain-to-strain variation in the assay detection limit. When reagents prepared with the polyclonal Ab20920 were used, the sensitivity of the assay was only 70%, rendering it inadequate for any clinical application (II). The reason underlying the poor sensitivity was obviously the low affinity of the polyclonal antibody. This is similar to the weaknesses recognized in association with the use of the first-generation slide agglutination tests for identification of *S. aureus* strains. Many MRSA strains fail to produce agglutination in these tests, which detect only bound coagulase and protein A (van Griethuysen, et al., 2001). The agglutinin-negative strains are known to possess a polysaccharide capsule which may physically mask cell surface structures (Fournier, et al., 1989). Accordingly, the polyclonal antibody was abandoned for the superior monoclonal reagents. The assay reagents prepared with both of the monoclonal anti-*S. aureus* antibodies (AM01227PU-N and BM3066X) provided equally high sensitivity and specificity in a limited collection of well-defined MRSA, MSSA, and CoNS strains (II). However, reagents prepared with the latter antibody offered superior performance in terms of SBR and measurement time requirements. Consequently the monoclonal anti-*S. aureus* BM3066X antibody was chosen to be used later as the immunoreagent in recognition of pure cultured MRSA colonies (III) as well as in the TPX MRSA screening assay (IV).

### 6.1.4. Detection of methicillin resistance by selective antimicrobials

Initially, oxacillin was used as the penicillinase-resistant beta-lactam agent to inhibit the growth of methicillin-susceptible bacteria (I). The decision to use cefoxitin (II) as the antimicrobial agent in the following studies was provoked by the results of Felten et al. (Felten, et al., 2002), who showed that cefoxitin might be superior to oxacillin as a selective agent for phenotypic testing of MRSA. A single concentration of 4 mg/L was able to inhibit the growth of all MSSA strains while MRSA and methicillin-resistant CoNS strains remained unaffected. In direct screening of colonization samples (IV) cefoxitin was combined with the monobactam aztreonam to even more effectively suppress the growth of other bacteria potentially affecting the fluorescence signal levels.

All beta-lactam antibiotics, such as oxacillin, cefoxitin and aztreonam, induce lysis of dividing susceptible bacteria and release intracellular antigens in the process. A higher concentration of soluble antigens allows a more constant distribution, which minimizes the decreasing effect on signal average by microparticles without any bound cells. Accordingly, soluble antigens released in the presence of antimicrobial agents from

dying bacteria cause the fluorescence signal level to rise with time even though the size of the living bacterial population is decreasing. Here, the hypothesis was supported by the effects of lysozyme treatment: lysozyme-induced lysis of bacteria increased absolute fluorescence signal levels by 30% (II).

### 6.1.5. Effects of coexistent microbes on TPX MRSA assay results

Measurement of sample turbidity has been widely used in automated antimicrobial susceptibility testing, but all of these techniques have relied on isolation of the bacterial strain to prevent the non-specific effects on turbidity caused by other microbes. The preliminary theory was that the TPX assay may offer an accurate quantitative measure of the amount of a specific bacterium within a sample, which is not significantly affected by the growth of other microbes. The specific fluorescence signal is measured when a microparticle coincides with the focus while the liquid background fluorescence, caused by unbound tracer and autofluorescent molecules, is recorded when there is no microparticle in the focus. The growth of any organism causes scattering and absorbance, increasing the turbidity of the sample. As both the specific fluorescence signal and the liquid background signal decrease in respect to increasing turbidity, a ratio of these signals should be compensated to a certain degree.

In the present study the effects of co-existing microbes on the TPX assay ability to monitor S. *aureus* growth were evaluated under experimental conditions. The assay was found tolerant to high excesses of CoNS. A 35-fold excess of *S. epidermidis* compared with *S. aureus* had no significant effect on the assay performance in monitoring *S. aureus* growth, although a very high, 350-fold excess, was found to significantly lower the absolute fluorescence signal levels (I). Moreover, specimens with artificial comingled bacteria (MRSA together with MSSA or CoNS) were not found to endanger the assay performance (II). Combined, these results implied that the MRSA assay might be applicable to direct screening of MRSA from colonization samples, and plausibly, also from other non-sterile clinical specimens.

### 6.1.6. Characterization of a growth [F(t)] curve typical of MRSA

Early in the study, one major goal was to discover and illustrate a growth [F(t)] curve typical of MRSA in the TPX assay conditions. Large inoculums of susceptible *S. aureus* cells release antigens into the medium and cause a proportional rise in the fluorescence signal level. As a consequence, MRSA strains cannot be reliably differentiated from the MSSA strains by the absolute fluorescence signal values alone. Instead, the results of the present study show that a steep rise in the fluorescence signal level at some point during a measurement (a high $\max\Delta F/\Delta t$ value) is characteristic of the growth of MRSA (II).

Considerable study-to-study variations in immunoreagent performance were observed during this work. In Study III the maximal fluorescence SBR achieved with reagents prepared with the monoclonal anti-*S. aureus* BM3066X antibody was only half of that reached in Studies II and IV. It was supposed that either the antibody or the fluorescent label had lost activity during storage or the labeling process. A lower SBR reduced absolute signal levels for both MRSA-positive and negative samples; accordingly, the assay was still able to differentiate between the 2 groups. It was due to the lower breakpoint max($\Delta F/\Delta t$) value defined for Study III that appropriate MRSA test results were provided. These results demonstrate that the breakpoint max($\Delta F/\Delta t$) has to be empirically re-evaluated whenever a new set of reagents or assay settings are adopted.

### 6.1.7. Grounds for false-positive and false-negative assay findings

Many factors may underlie MRSA false-positive and false-negative TPX assay results. Awareness of these pitfalls is essential for the prevention of as many as possible of these false test results. A high MSSA inoculum may yield a growth [*F(t)*] curve indistinguishable from that generated by MRSA, leading to a false-positive test result. This can be avoided by bearing in mind that an initially high fluorescence signal is an indication of an inconclusive screening test result. Conversely, due to the hook effect a *F(t)* curve determined for a high MRSA inoculum, initially above the assay dynamic range, may not show the characteristic steep rise in the fluorescence signal level, leading to a false-negative TPX test result. At hook, the saturation of the binding reagents leads to the descending part of the dose-response curve. These potential difficulties can be avoided by including several duplicates of the sample at different levels of dilution. On the other hand, overdilution of a sample yields an initial amount of MRSA considerably beneath the assay detection limit. To avoid a false-negative test result in such cases a longer measurement time is needed. These findings illustrate that a trade-off exists between assay performance and throughput capacity.

Further, a false-positive TPX test result may be caused by methicillin-resistant CoNS strains, as some CoNS strains are known to share cell surface antigens with *S. aureus*, causing false-positive reactions also in commercial *S. aureus* agglutination tests (Blake & Metcalfe, 2001). Then again, it is likely that microbes present in the samples sometimes disturb the TPX fluorescence measurements even if they are not recognized by the specific immunoreagents. It is imaginable that an excess of coexistent microbes in a colonization specimen could produce MRSA false-negative results probably by competing for nutrients, by increasing turbidity, and by nonspecific binding of the immunoreagents. False-negative TPX test results may also be caused by the failure of the applied antibody to recognize specific MRSA cell wall structures which are not exposed on the surface of the bacterial cell (Fournier, et al., 1989; Fournier, et al., 1993).

### 6.1.7. Assay settings

The above findings underline the importance of selecting proper assay settings to secure a satisfactory assay performance. The use of several replicate wells offers statistically more reliable results, though at the cost of increased reagent consumption, decreased assay throughput capacity, and increased labor input. Replicate wells were used only in the pre-thesis experiments while longer measurement times per assay well, i.e. more particle signals available for statistical analysis were later utilized.

At the outset of these studies, multiple sample dilutions were used to avoid potential errors associated with high amounts of staphylococci (I, II). The standardized low-density inoculum eliminated the need for multiple dilutions and allowed a high assay capacity (III). MRSA colonization samples contain unknown and highly variable amounts of bacteria. When colonization samples were screened (IV), 1 dilution of each sample was included to reduce potential false test results caused by high bacterial loads.

Dry-chemistry plates were adopted to allow a highly simplified 1-step assay protocol. It was shown that the plates could be stored for up to a month at +4°C without a loss of reagent functionality (II). In a clinical application such ready-to-use plates could be provided for the end-user to reduce the required laboratory personnel training level.

To prevent evaporation and spilling of potentially infective assay reagent mixtures the assay wells have to be sealed. When the experiments were duplicated with a highly gas-permeable plate-sealing tape instead of a regular tape, the assay performance did not improve (II). Hence, the cheap and more rugged plastic films were retained.

The temperature inside an operating TPX plate reader is held at approximately +32°C by heat production from the reader components. It has been reported that phenotypic susceptibility testing might provide more reliable results if performed at such relatively low temperatures (Cauwelier, et al., 2004). Active temperature control is not available in current TPX plate readers.

## 6.2. Applicability of the TPX assay for detection of MRSA in a large strain collection

On account of the limited set of isolates tested in Study II it was considered plausible that *S. aureus* strains not recognized by either of the 2 monoclonal antibodies used might be found in a wider scale testing. It was also deemed possible that some MRSA strains might be inhibited by the TPX test conditions. To evaluate the applicability of the TPX assay for recognition of a wider variety of MRSA strains, a large collection of

clinical MRSA isolates representing practically all different epidemic types recognized in Finland between 1991 and 2009 was tested in addition to sporadic MRSA, MSSA and CoNS isolates.

Based on the results of this assessment the TPX assay proved both sensitive (97.9%) and specific (94.1%) in identification of MRSA, with an adequate PPV (98.4%) and NPV (92.2%) when reagents were prepared with the monoclonal anti-*S. aureus* BM3066X antibody. Of the 386 Finnish MRSA isolates, only 8 (2.1%) gave false-negative TPX test results. Although *mecA*-positive, 1 false-negative isolate had a wide cefoxitin inhibition zone diameter, thus probably being unable to grow in the presence of 4 mg/L cefoxitin. The remaining false-negative isolates had resistant phenotypes in the cefoxitin disc diffusion test and were most likely not recognized by the monoclonal antibody. Three of the MRSA false-negative isolates produced positive MRSA test results when they were re-evaluated employing alternative reagents prepared with the monoclonal anti-*S. aureus* AM01227PU-N antibody. It is conceivable that a combination of 2 or more monoclonal antibodies could be used to improve the assay performance. Such assays are readily supported by current TPX plate readers. However, an expected improvement in the assay sensitivity would be modest in comparison to the increased assay complexity.

Of the 6 false-positive MSSA and CoNS isolates, 2 CoNS isolates exhibited high $\max(\Delta F/\Delta t)$ values. These CoNS isolates were able to grow in the test conditions and most likely had a significant cross-reactivity with *S. aureus*-specific immunoreagents. This assumption is supported by previous findings on shared cell surface antigens between some CoNS strains and *S. aureus* (Blake & Metcalfe, 2001). Considering the findings presented here, it was deemed likely that in very low MRSA prevalence settings a majority of MRSA-positive screening test results would be caused by MSSA or CoNS while false-negative screening test results would be rare.

The convincing assay performance in identification of this epidemiologically wide-ranging strain collection clearly illustrates that the TPX MRSA assay is tolerant to wide biological variation. Alarmingly, a trend towards a broader variety of new epidemic strains has been recently observed in Finland (Sissonen, et al., 2009). This of concern since the existing real-PCR tests may fail to identify emerging new epidemic MRSA clones, such as those harbouring the *mecC*. Inefficiency of these tests in settings with an abundance of circulating epidemic strains has been perceived in our country (Sissonen, et al., 2009) as well as in certain other environments (García-Álvarez, et al., 2011). It is notable that only 1 isolate representing 1 Finnish epidemic MRSA strain remained undetected here by the TPX MRSA assay, whereas all other isolates of epidemic strains were correctly identified. These findings underscore that the function of the TPX assay may remain uncompromised in environments with rapidly emerging new MRSA strains.

## 6.3. Applicability of the TPX assay for direct screening of MRSA colonization samples

Finally, the applicability of the TPX methodology for direct screening of MRSA colonization samples was evaluated (IV). The results showed that all patients positive for MRSA by conventional methods were positive also by the TPX assay, indicating that the sensitivity of the MRSA screening assay in recognition of MRSA-colonized patients was 100%. The sensitivity was lower, 89%, when it was calculated per a colonization sample. However, in each of the 4 patients with MRSA false-negative test results, only 1 colonization site was defined as negative while the results of more than 1 site were usually MRSA true-positive. Therefore, a false-negative screening result at 1 colonization site did not threat the recognition of that patient as MRSA-positive.

It is evident that further efforts should be made to improve the TPX assay performance in screening of MRSA colonization. Already in this study aztreonam was for the first time added to cefoxitin to inhibit the growth of an even wider spectrum of competing microbes at various body sites. The targets of aztreonam were gram-negative bacteria usually present in perineal and inguinal flora. Moreover, 1 dilution of each sample was included in the TPX assay to hinder potential errors associated with high amounts of MRSA or MSSA. Two wells per colonization site granted an assay capacity of 48 samples per test run. Later generations of TPX plate readers (mariPOC®, ArcDia Ltd) also incorporate random access capacity.

After a test run, the selectively enriched TPX reaction mixtures were collected for confirmation and further studies by conventional methods. Initially, the aim was to use conventional enrichment culture as the only reference method for the TPX MRSA assay. However, since the enrichment culture and the TPX assay were performed on 2 different swabs, it was possible that MRSA was sometimes present in only 1 of the 2 swabs, leading to contradictory assay results. In case of discrepancy between the 2 methods, the result of the culture of the TPX reaction mixture was taken into account to confirm either result.

In several cases, MRSA could not be isolated from the TPX assay reagent mixture through both the enrichment culture and the TPX assay were positive. This indicates that the test conditions were not optimal for the identification of MRSA from the TPX reaction mixtures. The reaction mixtures were cultured overnight on chromogenic agar. It has been shown earlier that the ability to detect MRSA may be significantly improved by prolonging the incubation time to 48 h (Han, et al., 2007). A larger sample volume could also improve the recovery rate in the future. The 96-well plate format readily allows more than 3 times larger sample volumes. Adoption of a lower density plate format would allow even larger sample volumes, yet with loss of throughput capacity.

The assay result was inconclusive, if the TPX detector was unable to locate enough of microparticles within the reaction mixture for a reliable fluorescence measurement. An inconclusive test result signifies that the specific colonization site cannot be defined as MRSA-positive or negative by the TPX assay. The clinician should then be advised that application of some other method is indicated. In none of the persons included in this study did an inconclusive TPX result from any site have an effect on the final designation of that person as MRSA-positive or negative based on the results from other colonization sites.

A majority of the inconclusive test results were recorded from throat and perineal samples. Some of the perineal samples were highly turbid causing absorption and scattering of the excitation light. The throat samples commonly contain viscous mucus, which forms droplets within assay reaction mixtures and on well bottoms. These were thought to act like miniature lenses dispersing the focused excitation light, thus preventing microparticle location within reaction mixtures. An emulsifier such as polysorbate 20 (Tween 20) might be used to prevent formation of droplets. Tween 20 also reduces nonspecific antibody binding and it has been widely used in previous applications of the TPX immunoassay methodology. Unfavorable effects of an emulsifier on the MRSA growth or on *S. aureus* antigen expression seem unlikely in usually applied concentrations.

Although so far not the best possible, the performance of the TPX MRSA appears to be acceptable especially when wide-scale MRSA screening is required, e.g., in epidemic situations. The TPX methodology could be used, in the future, to provide preliminary results for a clinician along with selectively enriched reaction mixtures for further conventional testing. The results are obtained quite rapidly: the median time required for confirmation of a true-positive TPX MRSA screening test result was 3 h and 26 min. Thus, one can hypothesize that with suitable modifications, the TPX methodology could be implemented at some point instead of the current broth enrichment step to provide the final results at an earlier time. Costs and workload associated with maintaining hospital infection control may be reduced by this high-throughput point-of care compatible assay. Moreover, rapid screening test results may be helpful when the clinical situation of a patient is contemplated, in order to choose an optimal empiric antimicrobial treatment even right from the beginning. As an example, a positive MRSA screening test result from a throat sample in a pneumonia patient suggests that the therapy should include an anti-MRSA agent. Similarly, a positive screening result from any body site suggests that in suspected invasive infections the therapy should include vancomycin or daptomycin. In addition, the TPX methodology may also provide a tool to screen for MRSA directly from other clinical samples, e.g., abscesses or postoperative wounds, to ensure the rapid commencement of appropriate antimicrobial therapy.

## 6.4. Benefits afforded by the use of the TPX MRSA assay

This doctoral thesis provides data on many qualities in favour of the TPX MRSA methodology. As the last point of the discussion of the TPX MRSA screening assay, the main advantages provided by this technique are summarized. First, the TPX assay is equipped with a high-throughput capacity, as up to 70 pure cultured samples were analyzed per a test run. To provide an optimal performance the number of samples tested in a single run was settled on 48. Second, after the procedure, the selectively enriched reaction mixtures can be recovered from the wells for further studies by conventional phenotypic and molecular methods to confirm the TPX assay result, when needed. Also, the strain picked from the assay well can be referred to antimicrobial susceptibility testing and *Spa* typing, which are regularly performed on all new MRSA findings in Finland. Third, the average time required for obtaining an MRSA true-positive TPX test result was 8 h from pure cultures and 3 h 26 min from MRSA colonization samples. The duration is tolerable considering that the commonly used routine phenotypic techniques require a minimum of overnight incubation for primary isolation of the bacterial strain. Admittedly, the real-time PCR assays are able to identify MRSA within a considerably shorter time but they are not practicable outside research laboratories. Quite the opposite, the automated ArcDia TPX microfluorometer was originally developed for use in point-of-care settings. Relatively small and inexpensive instrumentation, including robust microchip lasers, are utilized in the TPX device. Also the amounts of reagents consumed are small and the prices reasonable, contributing to low cost per analysis. What is more, the procedure is technically undemanding, thereby decreasing the workload of the employees. The single-step procedure is feasible without any highly trained laboratory personnel or sophisticated laboratory facilities. One can foresee that the TPX MRSA assay might be realistic one day also outside major laboratories, e.g., in emergency rooms, hospital wards, and even nursing homes.

## 6.5. Future aspects

In the present work, a number of issues regarding e.g., the assay settings were not fully resolved, and should be examined by further studies. The next intended goal of this research line will be making efforts to enhance the performance of the TPX MRSA screening assay. This can be commenced by comparing the effects of a variety of test conditions including different assay protocols and reagents as well as altered sample groundwork on the test results. There are other tempting options for application of the TPX method in bacteriology. In all probability, simultaneous testing of *S. aureus* for resistance to multiple antimicrobial agents, e.g., vancomycin, clindamycin and fluoroquinolones could be implemented by a straightforward extension of the current assay matrix. One can hypothesize that such a screening assay might be helpful in catching e.g., emerging MRSA strains with reduced susceptibility to vancomycin for further studies by appropriate methods. The cost would be an increase in the assay complexity with a concomitant reduction in the throughput capacity.

The TPX-based approach could be readily utilized also for detection and antimicrobial susceptibility testing of other clinically important MDR microorganisms. A single accessible cell surface target antigen is required for application of a simple assay procedure. The target could be either a species-specific or resistance- or virulence-associated cell structure. Further, an assay could be tailored to detect antigens released from lyzed bacterial cells in order to measure the rate of cell death in the presence of a bactericidal agent.

Alternative sample forms such as urine or stools could be tested as well. Some form of pre-treatment would probably be required for the latter to produce a transparent liquid sample for testing.

In principle, this kind of assay could be implemented also by some other separation-free assay technology platforms that would allow continuous or periodic quantitative measurements on a growth-sustaining environment. Fluorescence correlation spectroscopy might be an example of such a technique.

# 7. SUMMARY AND CONCLUSIONS

The basis and motivation for this doctoral thesis introducing a novel TPX-based MRSA screening methodology was established by the discovery that the growth of bacterial cells can be initiated and sustained in a TPX instrument and that the growth process is not significantly disturbed by periodical TPX measurements of the amount of specific bacterial antigens in the reaction mixture. A further essential invention was that the TPX method is suitable for antimicrobial susceptibility testing. These preliminary experiments provoked the idea that this same study concept could lead to a new innovation: construction of a TPX-based assay for the phenotypic screening of MRSA directly from clinical colonization samples.

Setting up a novel diagnostic assay provided many challenging issues to be addressed and solved. To begin with, the TPX assay principle had to be proven valid for the recognition of MRSA. The postulation was that in the TPX study conditions, MSSA and methicillin-susceptible CoNS do not grow under selective antimicrobial pressure, while the growth of MRSA is recognized by *S. aureus*-specific antibodies. A steep rise in the TPX fluorescence signal level at some point during a measurement was deemed characteristic of the growth of MRSA. Availability of an optimal antibody was verified as a major prerequisite for a successful assay procedure. The requirement was high sensitivity for *S. aureus* with only minimal cross-reactivity with CoNS. As a phenotypic method, the TPX assay was designed for screening purposes. Currently, a definite identification of methicillin resistance in any *S. aureus* strain should still be based on the detection of the presence of the *mecA* (or *mecC*) gene.

In this thesis, the applicability of the TPX assay for the detection of pure cultured MRSA isolates was evaluated in a large collection of clinical MRSA, MSSA and CoNS isolates. Included were practically all different epidemic MRSA strains identified in Finland between 1991 and 2009. The TPX assay was found to be both sensitive (97.9%) and specific (94.1%) in this epidemiologically variable population, illustrating that the TPX method is tolerant to wide biological variation.

It is of note that in certain environments, new MRSA strains have recently emerged, which cannot be identified by the existing real-PCR tests. Also in Finland, there has been a trend towards an even wider repertoire of epidemic strains. The convincing assay performance in identification of the Finnish epidemic MRSA strains underlines that the TPX assay has potentials for reliable functioning also in settings with rapidly emerging new MRSA strains.

In addition to the possible presence of MRSA, colonization samples of suspected MRSA carriers contain a number of other bacterial agents. These include especially CoNS and MSSA strains. It was shown early in this study that under experimental

conditions, MRSA specimens with these co-mingled bacteria do not jeopardize the TPX assay performance. This assumption was later verified by the successful recognition of MRSA straight from colonization samples of previously identified MRSA carriers: all patients positive for MRSA by conventional methods were defined here positive also by the TPX assay. These results enhance the confidence in that the TPX methodology is suitable for direct screening of MRSA colonization samples without any prior steps of isolation.

In this study, many benefits associated with the use of the TPX technology became manifest. The TPX MRSA assay had a high-throughput capacity rendering the technique suitable for wide-scale screening purposes. The median time required for the confirmation of a true-positive TPX MRSA screening test result was reasonable. Developed for use in point-of-care settings, the automated ArcDia TPX microfluorometer employs small and inexpensive instrumentation. Moderate prices of the reagents consumed also contribute to cost-effectiveness. Refined facilities or highly trained personnel are not needed to operate the single-step procedure. Based on these data, one can envision that the use of the TPX MRSA assay might be realistic one day also outside major laboratories.

Collectively, the findings presented in this thesis suggest that the TPX MRSA screening assay could be used, in the future, to provide preliminary results for a clinician along with selectively enriched reaction mixtures for further conventional testing. One can anticipate that with some modifications the assay could be adopted instead of an existing broth enrichment step to avoid additional delays to the final results. This can potentially mean that contact isolation of TPX screening test-negative suspected MRSA carriers with no previously recognized MRSA positivity could be discontinued earlier, thereby reducing the costs and burden associated with the containment of MRSA. Moreover, rapid colonization test results might help a clinician to begin an appropriate empiric therapy in MRSA carriers who develop infections. Another potential application for the TPX MRSA method, foreseeable in the future, might be screening for MRSA directly from clinical samples, such as abscesses and postoperative wounds, to warrant a rapid commencement of effective antimicrobial treatment. In so doing, the outcome of the patients with MRSA infections might be improved.

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Lieto, December 2012
Teppo Stenholm

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