Staphylococcus aureus **transmission**

-clinical and molecular aspects-

To my parents; in loving memory of my mother

Staphylococcus aureus **transmission –clinical and molecular aspects-**

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Staphylococcus aureus

transmission

-clinical and molecular aspects-

Verspreiding van *Staphylococcus aureus*

–klinische en moleculaire aspecten-

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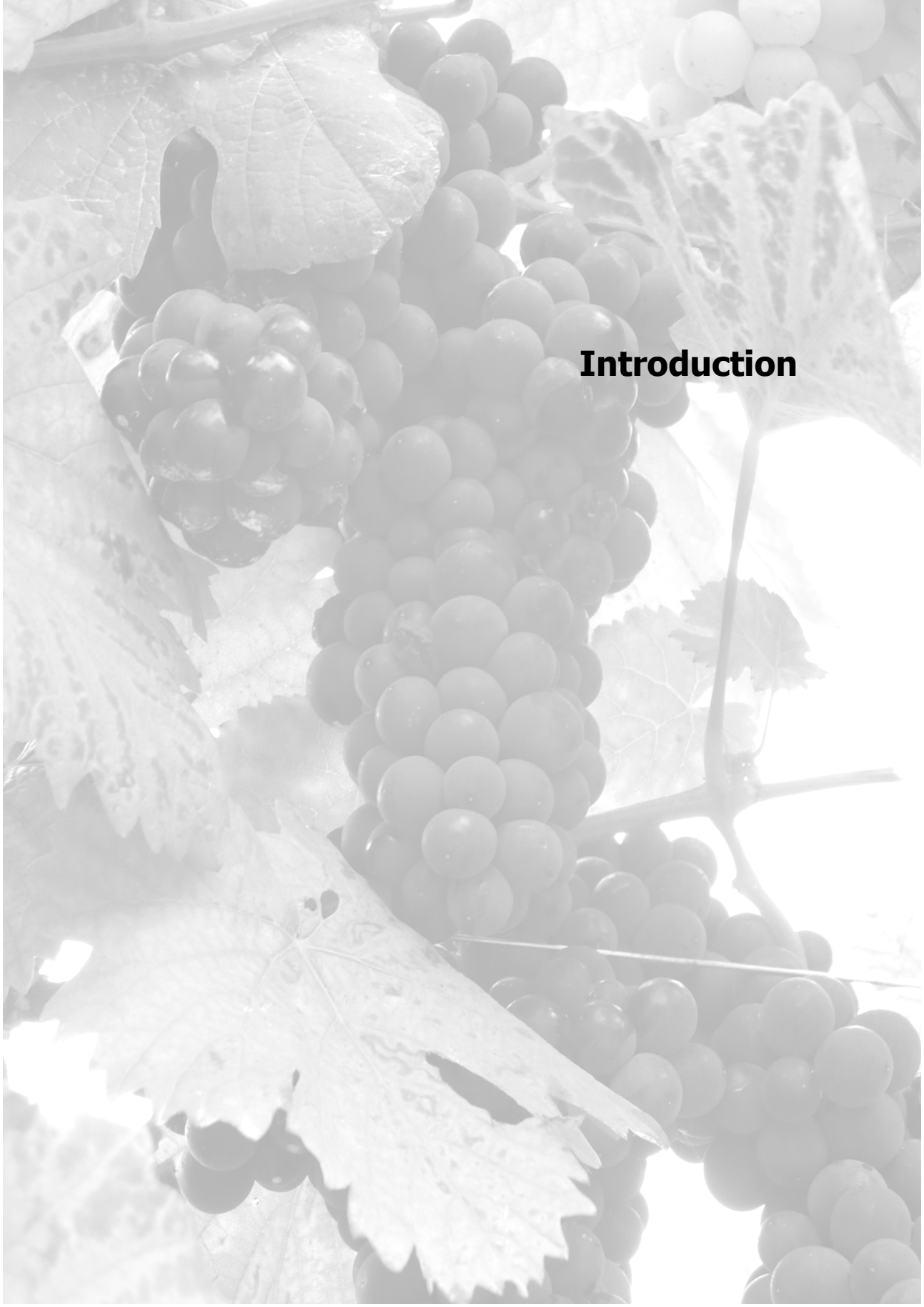

Introduction

Staphylococcus aureus

History

In 1881 Alexander Ogston named the bacterium commonly found in human skin infections *Staphylococcus*, inspired on its microscopic grape-like appearance [1]. Within three years Rosenbach added *aureus* tot the species name, due to its "golden" glow, when grown in pure culture [2].

In the days before the antibiotic era mortality due to *S. aureus* infection was extremely high. Smith et al. report a staggering 100% mortality due to *S. aureus* septicemia in adult females between 30 and 40 years of age [3]. In 1929, Alexander Fleming first reported on the bactericidal effect of penicillin, which was produced by the mould *Penicillium* [4]. Penicillin was found to be bactericidal to pyogenic cocci, such as *S. pyogenes, S. pneumoniae* and *S. aureus* and to the diphtheria bacillus. However, the first case of penicillin resistance in *S. aureus* was reported in 1944 [5] and the extensive use of penicillins during the 1950s selected for β-lactamase-producing *S. aureus* strains leading to an alarming increase in penicillin resistance [6,7].

The introduction of celbenin, better known as methicillin, was thought to be the definitive answer to penicillin-resistant *S. aureus*. Unfortunately, in 1961 the first cases of methicillin-resistant *Staphylococcus aureus* (MRSA) were reported [8].

Since the first account of MRSA, the prevalence has been increasing throughout the world. MRSA has become one of the most important pathogens in nosocomial infections [9-11]. Although MRSA is still predominantly a hospital related pathogen, the rising number of community-associated MRSA infections is an alarming predicament [12].

Antimicrobial resistance

Methicillin resistance

MRSA strains contain the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*), which carries the resistance gene for methicillin, *mecA* [13,14]. There are 5 SCC*mec* subtypes (types I–V), which vary in size from ~ 20 kb to ~ 68 kb. The smaller subtypes (I, IV, and V) encode only recombinase genes and the structural and regulatory genes for resistance to methicillin. They do generally not carry genes encoding resistance to non–β-lactam antibiotics [15]. *MecA* encodes for the penicillin-binding protein PBP2a, that has lost affinity for all ß-lactam antibiotics. Penicillin-binding proteins (PBPs) are membrane proteins whose normal function is to catalyze the cross-linking of the bacterial cell-wall [14]. PBP2 is the target protein of β-lactam antibiotics, leading to loss of cell-wall integrity. Expression of PBP2a therefore renders *S. aureus* resistant to all β-lactam antibiotics. However, resistance levels to β-lactam antibiotics differ between different MRSA strains, dependent on the levels of PBP2a production [16].

Glycopeptide resistance

Glycopeptides (e.g. vancomycin and teicoplanin) are the most important antimicrobials in the treatment of MRSA. However, the first report on reduced susceptibility to vancomycin (vancomycin intermediate susceptible *S. aureus*, VISA) of a clinical *S. aureus* isolate was published in 1997 [17]. Since 2002 at least 7 cases of vancomycin-resistant *S. aureus* (VRSA) have been reported [18]. *S. aureus* strains requiring concentrations of vancomycin of <4 µg/ml for growth inhibition as susceptible, those requiring 8 µg/mL to 16 µg/ml for inhibition as intermediate, and those requiring concentrations of >32 µg/ml as resistant [19].

Comparable with β-lactam antibiotics, glycopeptides inhibit *S. aureus* growth by preventing the assembly of the bacterial cell-wall. The *S. aureus* cell-wall is made up of peptidoglycan layers that consist of a

series of murein monomers, each of which has a d-alanine–d-alanine residue. The monomers are synthesized within the cell, transferred through the cytoplasmic membrane, and assembled into peptidoglycan outside of the cytoplasmic membrane by enzymes located within the membrane. Vancomycin inhibits bacterial cell growth by binding to d-alanine–d-alanine residues of the monomers and preventing the assembly of peptidoglycan outside of the cytoplasmic membrane [20].

The proposed mechanisms of resistance described for VISA and VRSA strains are distinctly different from each other. The primary factor that causes reduced susceptibility to vancomycin in VISA isolates is the presence of a thickened cell-wall with several more peptidoglycan layers, compared with non-VISA isolates. Vancomycin binds to the many d-alanine–d-alanine residues within the additional peptidoglycan layers and never reaches the surface of the cytoplasmic membrane to exert an effect on the synthesis of peptidoglycan [21].

VRSA arises due to the exchange of genetic material between bacteria. Acquisition of the *vanA* resistance gene most likely occurs via interspecies transfer of the *vanA* transposon, *Tn1546*, from a vancomycin-resistant Enterococcus (VRE) strain [22,23]. Resistance to vancomycin is achieved by the synthesis of a cell-wall precursor molecule that is truncated with the muropeptide sequence d-alanine-d-lactate, instead of d-alanine-d-alanine. The altered muropeptide has reduced affinity for vancomycin, thereby allowing peptidoglycan assembly [24].

The clinical significance of intermediate susceptibility to glycopeptides is debatable [18,25]. Charles et al. reported higher bacterial load and more persistent fever in VISA bacteremia compared to vancomycin-susceptible MRSA [26]. However, Fridkin et al. could not show a clinically relevant difference in infections by MRSA with or without reduced susceptibility to vancomycin [27].

The emergence of serious staphylococcal infections with reduced susceptibility to vancomycin highlights the need for more antimicrobial options with increased potency or enhanced bactericidal activity

against MRSA, VISA and VRSA [18]. Only two new classes have been introduced over the past few decades: the oxazolidinones and the cyclic lipopeptides [28]. Among the antimicrobial agents that have more recently been approved for clinical use, daptomycin (a cyclic lipopeptide), linezolid (an oxazolidinone) and tigecycline (a glycylcycline) have activity against Gram-positive organisms, including MRSA, VISA and VRSA. In addition, the new investigational compounds dalbavancin, telavancin, oritavancin, ceftobiprole, and iclaprim, have demonstrated in vitro activity against VISA and VRSA [29].

Epidemiology

General population

S. aureus is a normal mucanous and cutanous commensal in healthy individuals. Approximately 30% of the general human population will carry *S. aureus* in a cross-sectional assay, without threat to general health [30]. The population carriage of MRSA is low. A meta-analysis showed this percentage to be 1,3% of all *S. aureus* carried [31]. However, the results found in the different studies varied strongly (0.4%-7.4%). Healthcare associated risk factors are an important factor in the community carriage of MRSA. Prevalence of MRSA among people without these risk factors is <0.2% [31].

Hospitals

Carriage of *S. aureus* among hospitalized patients remains comparable to population carriage. However, hospital carriage of *S. aureus* severely increases the risk of development of a nosocomial infection [32]. A considerable difference between population and hospital carriage is the prevalence of MRSA. Studies monitoring hospital carriage and infection by MRSA worldwide show alarming MRSA prevalence rates of up to 60% in Argentina, the US and European countries, although low prevalence is found in northern European countries as The Netherlands, Norway, Sweden, Denmark and Finland (Fig. 1) [9,10,33-36].

Nosocomial infections

Patients admitted to a hospital are at a considerable risk of acquiring an infection. Overall prevalence of nosocomial infections is between 3.5% and 12% [37-44] and these infections lead to higher patient mortality and increased healthcare costs [45-47]. *Staphylococcus aureus* is one of the predominant pathogens found in nosocomial infections, especially in surgical site and catheter related infections [37,43,48,49].

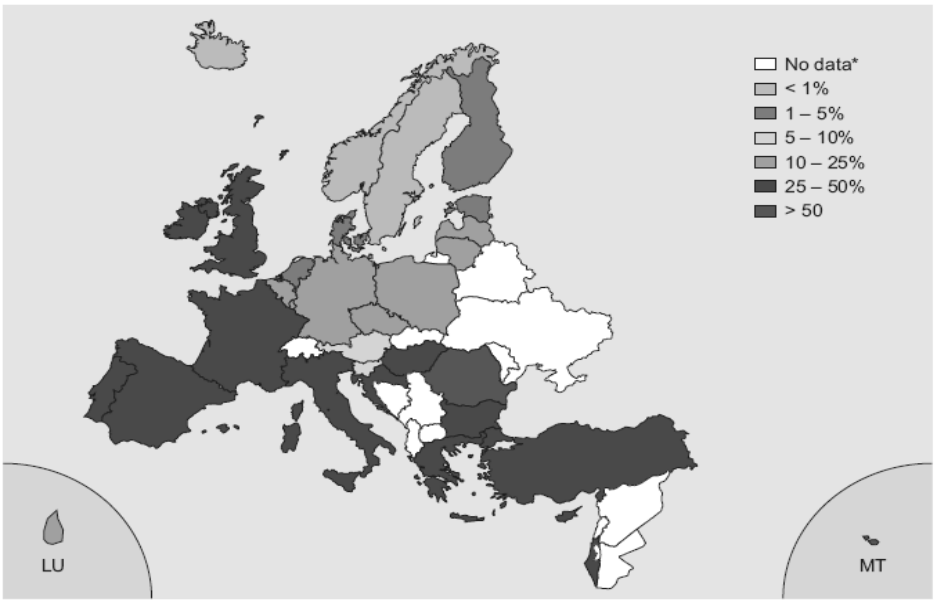

Figure 1. *Staphylococcus aureus*: proportion of invasive isolates resistant to oxacillin (MRSA) in 2006 (taken from EARSS annual report 2006)[10].

Up to 45% of all nosocomial infections occur during stay on an intensive care unit (ICU), although only 8% patients will be admitted to an ICU [50]. Thirty to forty percent of all patients admitted to an ICU will have or acquire an infection [41,51-53]. As in the general hospital, infection on the ICU leads to an increased length of stay, mortality and healthcare costs [54,55].
S. aureus is one of the most frequently cultured pathogens in ICU infections, accounting for up to 30% of these infections [44,51,56]. Surgical site, skin and soft tissue, bloodstream and respiratory tract are the predominant sites of *S. aureus* infections on an ICU [40,57].

Community acquired MRSA

The term community-acquired MRSA or community-associated MRSA infection (CA-MRSA) refers to an infection which originates in the community in contrast to hospital-acquired MRSA (HA-MRSA) infections. However, the definition of CA-MRSA remains a point of debate in terms of time to MRSA identification (varying from 24h to 72h in different studies), history and time of prior hospitalization (1 to 24 months) and exposure to healthcare associated risk factors. In

February 2005, the Centers for Infectious Disease Control and Prevention (CDC) defined CA-MRSA infection as: identification of MRSA in a patient with signs and symptoms of infection, either in the outpatient setting or within 48h after admission to a hospital, with no history of MRSA infection or colonization, no history of admission to a hospital or a nursing home during the previous year, and absence of dialysis, surgery, permanent indwelling catheters or medical devices that pass through the skin to the body [58].

CA-MRSA differs to HA-MRSA epidemiologically, genotypically and phenotypically [59]. CA-MRSA causes aggressive infections like suppurative skin infections and necrotizing pneumoniae in the young and healthy. The most well-known community-acquired MRSA virulence factor is PVL, which elicits tissue necrosis and may contribute substantially to the clinical findings of epidemic furunculosis and severe necrotizing pneumonia [60-63]. On the other hand, the *tst* gene that encodes toxic shock syndrome toxin 1 (TSST1), the toxin associated with the most typical staphylococcal toxic shock syndrome cases, is absent in the majority of community-acquired MRSA isolates, but other toxins capable of producing toxic shock syndrome-like illness have been identified [64].

Community-acquired MRSA strains harbor the smaller SCC*mec* type IV or V elements, which make it possible to grow faster and achieve higher infection burdens than nosocomial MRSA strains [65,66]. This property has been attributed to the extra metabolic burden that multiresistant bacteria have secondary to the synthesis of extra proteins during replication [67], and this may provide a selective advantage to community-acquired MRSA.

The emergence of MRSA infections in the community complicates the choice of antibiotic regimen to treat community onset infections. Generally, CA-MRSA is susceptible to more non-β-lactam antibiotics, especially if no health care associated risk factors are present [68]. However, CA-MRSA with healthcare associated risk factors have higher levels of resistance. Empirical therapy, especially in high prevalent CA-MRSA countries and in cases with health care associated risk factors, should be vancomycin, until antibiotic susceptibility for another antibiotic has been proven. Next to antibiotic therapy clinicians should consider non-antibiotic therapy for skin lesions due to CA-MRSA, as cutanous abscesses typically resolve with proper drainage and/or debridement alone, and collections left

without drainage in the setting of antibiotic treatment promote the emergence of resistance [69].

Colonization

S. aureus generally colonizes human skin and mucous membranes. The anterior nares are the most frequently site of carriage [70]. Approximately 30% of the general population will be colonized by *S. aureus* at one time point [30] and three types of carriership can be distinguished: persistent carriers (±20%), intermittent carriers (±30%) and non-carriers (±50%) [30,71-73].

Nasal colonization by *S. aureus* is one of the most important factors in the pathogenesis of nosocomial infections, mainly in surgical site and vascular device related infections [74-77]. However, the efficacy of prophylactic eradication of nasal *S. aureus* carriage in the reduction of the number of nosocomial *S. aureus* infections remains undecided [78-83].

Clonality of colonization

The underlying presumption of most studies is that nasal *S. aureus* carriage is clonal, especially in persistent carriers. This is mainly based upon an old review [70], which based results on phage-typing. Vandenbergh et al. studied a large group of patients over a 10 year period and concluded the opposite; only a small minority of patients carries one distinct *S. aureus* strain [73]. Other studies have shown polyclonal carriage within (non-)hospitalized patients [84,85] and even simultaneous MRSA and MSSA carriage in one patient. Lim et al. demonstrated intermittent and inconsistent MRSA carriage on different anatomical sites in ICU patients [86]. A possible explanation for the carriage of distinct strains is the large amount of antibiotics administered on ICUs, which can influence bacterial flora and clonality [87,88]. In addition, acquisition of *S. aureus* is influenced by antibiotic pressure [89-92]. Another explanation could be the generally poor health status in ICU patients, increasing the chance of acquiring a *S. aureus* [93,94].

ICU acquisition

As colonization by *S. aureus* is one of the most important predisposing factors in nosocomial infections, the acquisition of *S. aureus* is a vital area of attention in infection control. A patient admitted to the ICU can

acquire nasal colonization by *S. aureus* from different sources: endogenous (i.e. from other body sites), from the environment, from healthcare workers (HCW), from other patients (cross-transmission), or from outside the ICU (e.g. visiting family) [95-103].

Infection control measures

Contamination of hospital environment and equipment has been postulated as a possible source of acquisition [100,104-108], although transmission from the environment to a patient was relatively infrequent on the ICU [96,103].

It is not hard to imagine, that the role of HCWs in the spread of *S. aureus* is significant, especially on an ICU, where physical contact between nurse and patient is relatively frequent and extensive and the number of contacts with different physicians is high. The cohorting of HCWs to certain rooms or patients could be an important aspect in reducing ICU cross-transmission [109], but is hard to realize in an acute medical ward, mainly due to understaffing. In addition, nurse understaffing and increased workload correlate to a strong increase in *S. aureus* acquisition on the ICU [110,111].

The most important infection control measure regarding HCWs is hand hygiene compliance, as increase in compliance reduces the number of ICU infections [94,112-114]. However, HCW hand hygiene compliance is generally low in ICUs [115-117] and measures taken to increase compliance to hand hygiene are limited in efficacy and hard to implement [113,118,119]. Additionally, increased workload of nurses is inversely correlated to handwashing compliance [120].

Use of antibiotics prior or during ICU stay increases the chance of acquiring an *S. aureus* infection [89,99]. A reduction in the use of antimicrobials or implementation of an antimicrobial-prescribing improvement program reduces the number of *S. aureus* infections [121-123]. This infection control measure has unfortunately not been implemented in many ICUs, as it is necessary for each hospital to generate a specific antibiotic-use regimen, adjusted to local and regional epidemiology and patient- characteristics. This is a time-consuming and costly operation.

Isolation treatment of patients carrying MRSA reduces the number of MRSA acquisitions on an ICU [124-128]. However, an English study contradicts these results [129]. In addition, a meta-analysis could not show undisputable evidence for the correlation between isolation treatment of MRSA carriers and the reduction of transmission, mainly

due to poor methodology and inadequate reporting in the analyzed articles [130]. Single room treatment of all patients, contrary to isolation of proven carriers of MRSA, leads to low acquisition and cross-transmission rates of *S. aureus* and predominantly MRSA [131], although this is shown in one single center study so far.

Length of stay

Length of stay (LOS) is a widely accepted risk factor for the acquisition of MRSA. Unfortunately, in a number of studies this correlation has been shown for total LOS of patients in the ICU, including the period in which patients are colonized already [51,99]. In this period the patient is no longer at risk of acquiring a MRSA and should be left out of the analysis. Whether time to acquisition, or LOS at risk, is correlated to an increased acquisition rate is not clear [93,94].

Colonization pressure

The correlation between the number of colonized patients on an ICU and acquisition of pathogens has been shown for MRSA [99,132] and VRE [133]. However, this relation was not found in other studies [111,129]. These contrasting findings could be explained by a difference in definition and estimation of colonization pressure.

Cross-transmission

To which extent transmission of *S. aureus* from one patient to the other (cross-transmission) is responsible for acquisitions of *S. aureus* by patients on the ICU is strongly disputed. A German study shows an important role for cross-transmission in the acquisition of MRSA in a surgical ICU [102], but a number of other studies do dispute these findings [97,131,134,135].

Animal studies

S. aureus colonizes the skin and mucosae of human beings and several animal species and the anterior nares are the most frequently colonized site [70,136]. As nasal colonization is an important pathogenic factor for *S. aureus* infections, it is essential to be able to study variables like antimicrobial use and genetic variations in host and pathogen *in vitro* and *in vivo*. Human nasal colonization by *S. aureus* is strongly influenced by host predisposing diseases such as diabetes mellitus, haemodialysis, HIV and even obesity [136,137]; sufferers are more likely to carry *S. aureus*. In addition, a number of human genetic factors influence carriage of *S. aureus* [137], predisposing certain humans for persistent nasal *S. aureus* carriage. In addition to host factors, *S. aureus* can efficiently evade the human immune system upon colonization through a large number of virulence factors, compromising the effectiveness of host cellular response, reducing complement activation, inhibiting opsonization and even reducing host inflammatory response [138,139].

In order to study the significance of intrinsic pathogen factors for certain virulence characteristics, it is necessary to develop an *in vivo* study model, nullifying host traits as influential factors. To this end a number of murine and rat colonization and infection models has been developed [140-145]. These models are also often used for the assessment of antibiotic treatment efficacy.

Nevertheless, these models have clear limitations. Host specific immune response could differ strongly in animals and humans. Furthermore, bacteria are known to adapt to host specific characteristics through specialized virulence factors. The isolates used in these animal models are "human pathogens", introduced into a non-human environment. Although animal models are a necessary and invaluable tool to gain insight in the microbiology of *S. aureus*, conclusions drawn in animal studies should be extrapolated cautiously to the human situation.

Molecular typing

Molecular typing of pathogens can be performed for two main purposes. The first is to investigate the genetic composition of the pathogen in order to study resistance, virulence and other pathogen and strain specific characteristics [146]. The second aim of molecular typing is to assess clonality of strains at any level, to gain insight in the spread of different clones on a national or worldwide level, or to assess local spread and hospital hygiene levels [147].
To gain a better understanding of the relation between strains, which are isolated on one ICU, a number of typing methods has been introduced.

Phenotypic typing

Antibiogram

Susceptibility of strains to a range of antibiotics can be used to identify relations between different *S. aureus* isolates [148,149]. This method is phenotypical and crude. The method is low in discriminatory power and reproducibility.

Phage typing

An *S. aureus* isolate can contain temperate bacteriophages. The typing of *S. aureus* by applying an isolate to a plate containing test organisms to assess the presence or absence of these phages has been standardized [150]. A major disadvantage of phage typing is the large proportion of isolates, which is not typable (≤75%) [151-154]. *S. aureus* can be non-typable by phage typing for a number of reasons: 1. bacteriophages must first bind to a receptor on the bacterial membrane. This receptor can be absent. 2. *S. aureus* DNA may contain one or more restriction/modification systems, which can destroy bacteriophage DNA after injection in the bacterial cell, acting as a "DNA immune system". 3. the bacterial DNA may contain lysogenic phages, which may interfere with the replication of lytic bacteriophages. The overall value of phage typing is low.

Genotypic typing

PFGE

Pulsed-Field Gel Electrophoresis (PFGE) allows comparison of the complete genome of isolates. Therefore all strains are (in principle) typable by this method. The most frequently used restriction enzyme for PFGE of *S. aureus* is *SmaI,* although others have been used [155-158].

PFGE has high discriminatory power and has been proposed as gold standard for bacterial typing [151]. However, PFGE has a number of limitations. First, it is a time-consuming method [159]. Secondly, intercenter comparisons have been only partly successful [160,161] due to the fact that experimental conditions and interpretation of results must be standardized to obtain comparable results.

RAPD

Typing by Random Amplified Polymorphic DNA has been used to type *S. aureus* and all isolates are typable using this method. It is relatively inexpensive and quick. However, intercenter reproducibility is low [162] and discriminatory power is lower than in PFGE [163,164]. RAPD is not suitable as a long-term or reference method.

Spa-typing

The Staphylococcal *spa* gene encodes for protein A, an important virulence gene. Sequencing of the DNA of a polymorphic 24 kb variable-number tandem repeat within the *spa* gene can be used as a typing method in outbreak situation [165] and for phylogenetic analysis [166-168]. As *spa* typing is sequence based the reproducibility is high and all strains are typable [165]. It is less time consuming and cheaper than PFGE. However, discriminatory power is slightly lower compared to PFGE [168].

MLST

Multilocus sequence typing (MLST) is the most important typing method for the investigation of evolution and population structure of *S. aureus* an national and global scale. MLST is unambiguous and highly reproducible. The sequenced genes are 7 housekeeping genes, which are relatively stable within clonal lineage [169,170]. Discriminatory power is less than for PFGE. Unfortunately, MLST is

therefore not utilizable for outbreak and infection control analyses within healthcare facilities.

MLVA

Multiple-Locus Variable-Number Tandem Repeat Analysis (MLVA) is a PCR based typing technique [171]. Variable-Number tandem repeats (VNTRs) are chromosomal loci containing a variable number of tandem repeats and therefore differing in length. In *S. aureus* these VNTRs have been called Staphylococcal Interspersed Repeat Units (SIRUs), of which 7 have been selected for analysis [172]. Using PCR, these SIRUs can be analyzed for the number of repeats. MLVA is highly discriminatory and reproducible. In addition, it is a cheap and rapid method, which makes it suitable for both outbreak analyses and epidemiologic studies [173,174].

Microarray

Microarrays make it possible to study the presence and transcription of a large number of genes in one isolate at once [175-177]. Comparative Genomic Hybridization (CGH) is used to assess the presence and absence of chromosomal DNA fragments (i.e. genes) of a cell. Probes spotted on the microarray slides are either oligonucleotides or PCR fragments. DNA extracted from experimental isolates is labeled and hybridized to the slide. If the investigated DNA fragments are present in the experimental DNA, these fragments will hybridize to the DNA fragments on the slide. Under UV-light the labeled experimental DNA will become fluorescent. Microarray also allows the determination of a gene expression profile through Transcription Profiling (TP). TP assesses the presence of messenger RNA and is therefore in fact a phenotypic analysis. However, the level of transcription is only a rough estimate of the level of protein production. An mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. In addition, gene transcription is highly dependent on cell cycle phase and strongly effected by environmental changes, rendering TP unsuitable for genotyping. Using *proteomics* one can investigate the actual production of proteins and the way proteins can interact (e.g. cell signaling cascades). Using *metabolomics* one can investigate the metabolic processes in a cell, effected by proteins and enzyms, creating insight in the end-stage result of gene expression in a biological organism [178].

CGH is an effective (yet expensive) method to extensively genotype a bacterial isolate. Influences of cell cycle, environment and drugs on the transcription of genes can be investigated by TP, but proteomics and metabolomics are more important tools in this field of research.

Whole genome sequencing

To compare the full genetic assembly of different isolates it is necessary to sequence the complete genome of the isolates. Up to date 13 whole genome sequences of *S. aureus* have been undertaken and published in GenBank database (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Subtle genetic differences have been shown to have a major influence on virulence of *S. aureus* strains [179]. Whole genome sequencing is of course the most complete method of analyzing of the genetic diversity between isolates, but the vast amount of information gathered and the high price make this method impractical for outbreak and evolutionary analysis. In addition, it is hard to estimate the clinical relevance of subtle or small changes (e.g. point-mutations) in genetic build-up of one individual bacterial isolate.

Outline of this thesis

The acquisition, spread and clonality of *Staphylococcus aureus* on ICUs are important areas of attention in infection control and treatment. To better understand the different aspects of the ICU epidemiology of *S. aureus* and to assess cross-transmission of this pathogen a number of studies was undertaken. In addition, the strains collected in this study were used to evaluate different typing methods.

In **Chapter 2** the epidemiology of the acquisition and cross-transmission of *S. aureus* in the 6 participating ICUs (Utrecht, Lyon, Porto, Seville, Catania, Athens) in the *Staphylococcus aureus* Epidemiology and Transmission dynamics on Intensive Care units (SEPTIC) study is described. The main infection control measures and factors influencing acquisition are considered.
In **Chapter 3** the clonality of *S. aureus* carriage of patients in the SEPTIC ICUs is described. To assess genetic clonality MLVA and *spa*-typing were performed on all isolates.
In **Chapter 4** we describe the development of a murine bacterial transmission model for *S. aureus*. Based on a colonization model described by Kiser et al. [140], we modified the model to assess spread of both MSSA and MRSA between mice. The inoculated MSSA and MRSA are isogenic, except for a type IV SCC*mec* in WKZ-2 MRSA. The results of the whole genome sequences of MSSA WKZ-1, MRSA WKZ-2 and the SCC*mec* donor *Staphylococcus epidermidis* 07.1 are described in **Chapter 5.**
In **Chapter 6** we describe the results of a microarray study performed on a selection of the strains collected in the SEPTIC study. The main aim of the study is to assess the prospect of using microarray as a rapid, comprehensive and cost-efficient method for strain identification in infection control and hospital epidemiology.

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Acquisition and cross-transmission of *Staphylococcus aureus* on different European intensive care units

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Abstract

Objective: To study the acquisition and cross-transmission of *Staphylococcus aureus* in different ICU settings.

Design and setting: Multicenter cohort study. Six ICUs in six countries participated. During a three month period per ICU, all patients were swabbed nasally and perineally on admission and during stay. All collected *S. aureus* were genotyped by *spa*-typing and MLVA-typing for cross-transmission analysis.

Patients: 629 patients were admitted, of whom 224 were colonized by *S. aureus* at least once during stay (22% MRSA). 316 patients, who were *S. aureus* negative on admission and had at least one follow-up culture swab taken were eligible for acquisition analysis.

Results: In total 45 patients acquired a *S. aureus* during ICU stay (31 MSSA and 14 MRSA). Several factors believed to affect the acquisition of *S. aureus* were analyzed in univariate and multivariate analyses: hand disinfectant use, colonization pressure, beds per ratio, antibiotic use, length of stay and ICU setting. Colonization pressure and the number of beds per nurse correlated with the acquisition of both MSSA and MRSA. ICU-setting was related to MRSA acquisition only and hand disinfectant use to MSSA acquisition only. In 18 acquisition cases (40%) cross-transmission from another patient was possible.

Conclusions: Colonization pressure, number of beds per nurse and treatment of all patients in single rooms correlate to the number of *S. aureus* acquisitions on an ICU. Hand disinfectant use was correlated to MSSA acquisition, but not to MRSA acquisition. Patient-to-patient cross-transmission was comparable in numbers between MSSA and MRSA.

Introduction

Nosocomial infections dramatically increase mortality rates on intensive care units (ICUs) and *Staphylococcus aureus* is one of the most important pathogens in these infections, accounting for up to 30% of cases [1-3]. In addition, nosocomial pathogens often acquire a high level of antibiotic resistance. Methicillin resistance of *S. aureus* was first reported in 1961 [4] and subsequently spread throughout hospitals worldwide [5]. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) differs strongly between countries [6].

Colonization by *Staphylococcus aureus* is a major risk factor for developing a nosocomial infection [7]. The risk of becoming colonized during stay in an ICU is dependent on a number of variables, among which colonization pressure [8] and antibiotic use [9-13]. Infection control measures have been implemented worldwide to prevent transmission of MRSA, but there is clearly no consensus concerning the importance of each independent measure [14-18].

Possible sources for acquisition of *S. aureus* are other patients, health care workers (HCWs) and the ICU environment [19-27]. Endogenous reservoirs of *S. aureus* could also play an important role in what may falsely appear an acquisition of *S. aureus* in ICU patients [28]. Consensus on the importance of patient-to-patient cross-transmission of *S. aureus* has not been reached as study results have been highly variable [23,24,29-32].

These results were mainly obtained in single center studies and assessed MRSA only. It is unclear whether these considerable variations in acquisition and cross-transmission rates are due to differences in *S. aureus* prevalence and infection control measures or merely reflect variations in study protocol.

The aim of the current study was to evaluate baseline acquisition and cross-transmission rates of both methicillin-susceptible *S. aureus*

(MSSA) and MRSA in different ICU settings. To this end we performed a multicenter study that compared the prevalence, acquisition and spread of MSSA and MRSA in six European ICUs. Patient-to-patient cross-transmission was studied by genotyping of all strains by MultiLocus Variable number of tandem Repeats Analysis (MLVA) and *spa*-typing [33-36]. MSSA and MRSA were analyzed separately due to the important differences in general epidemiology between the two strains.

Methods

Setting.

The study was undertaken in 2006 in 6 ICUs in 6 university and teaching hospitals (total 77 beds) during a period of 3 months per center. The centers were located in The Netherlands, France, Portugal, Spain, Italy and Greece.

The following information on ICU setting was registered: number of beds, amount of hand disinfection used per year, single room versus open ICU treatment, isolation treatment options.

Center 1 is an 18 bed ICU, with 6 isolation rooms under negative pressure. Four isolation rooms have an antechamber for changing and disinfection of personnel in cases of patient isolation due to resistant pathogens (including MRSA). The other 10 beds are in one large room. Center 2 has 15 single rooms without antechamber. Nurses are cohorted during daytime. Center 3 has a 14 bed open ICU with no cohorting of nurses. Center 4 has a 15 bed open ICU. No cohorting is implemented. Center 5 is an 8 bed ICU without isolation room. Nurses are not cohorted. Center 6 is a 7 bed ICU, without cohorting. Centers 3, 4, 5 and 6 do not possess or rarely use isolation rooms.

Microbiological samples.

All patients admitted to the ICUs within the study period were included in the study. Nasal and perineal swabs were taken within 48h of admission and on discharge. During ICU stay, all patients were swabbed nasally and perineally twice a week on fixed days. Clinical *S. aureus* samples were also collected for analysis. Samples were collected and processed by the dedicated investigator. Swabs were cultured for 24h at 37°C in 4 ml trypsin soy broth (TSB) with 5 mg/L aztreonam. Samples were plated on mannitol salt agar (MSA) and blood agar (BA) plates and incubated for 48h at 37°C. Species identification was obtained by standard methods. All collected *S.*

aureus were transported to the principal investigator on Amies transport swabs and kept at 4°C.
All strains were examined by a triplex PCR for 16S rRNA, *nuc* and *mecA* genes [37]. DNA was isolated using a Nucleospin Tissue Kit (Machinery-Nagel) according to manufacturer's protocol.

Genotyping.
All isolates were genotyped by the principal investigator in Utrecht using MultiLocus Variable number tandem repeat Analysis (MLVA) and *spa*-typing. MLVA was performed by PCR of 6 variable number tandem repeats (VNTR) [38,39]. The analysis was performed as described previously by Ikawaty et al [40]. *Spa*-typing was performed using 5'-ACGAGTAGTGCCCTTTGCTT and 3'-GCTCAAGCACCAAAAGAGGA PCR primers (Invitrogen, Breda, the Netherlands) or 5'-TAAAGACGATCCTTCGGTGAGC and 3'-CAGCAGTAGTGCCGTTTGCTT (Isogen Life Sciences, IJsselstein, the Netherlands) PCR primers. Subsequent DNA sequencing was performed using chain termination method (Baseclear BV, Leiden, the Netherlands). Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze obtained sequences and assign *spa*-types. Novel *spa*-types were submitted online to the Ridom database (www.SpaServer.ridom.de).

Prevalence and acquisition.
The prevalence of *S. aureus* was defined as the percentage of patients with at least one *S. aureus* positive culture swab during ICU stay.
Acquisition of *S. aureus* was determined in patients who were negative for *S. aureus* on admission to the ICU (at risk patients) and was classified as ICU-acquired if the first positive culture for *S. aureus* was obtained at least 48h after ICU admission. Acquisition rate was defined as the percentage of at risk patients that acquired

S. aureus and remained on the ICU long enough to have at least one follow-up culture swab taken.

Patient information.

Patient information obtained included date of birth, date of admission and discharge and antibiotic treatment regimen.

ICU length of stay (LOS) was calculated for each patient. Length of stay at risk (LOS at risk) was defined as the number of days prior to acquisition of *S. aureus*.

Infection control measures.

In order to estimate hand hygiene levels, the total amount of hand disinfectant used in the study period was determined. The number of hand disinfectant doses used per bed per day was calculated by dividing the total amount used by the number of beds. One hand disinfectant dose was defined as 3 ml of disinfectant for all types (soap, liquid or gel).

Single room and isolation treatment procedures were classified in two levels of treatment (ICU set-up): 1) all patients were treated in single rooms; 2) patients were treated in an open bay setting.

Colonization pressure.

Average colonization pressure was defined as the mean percentage of patients colonized by *S. aureus* during the whole study period. Pre-acquisition colonization pressure was defined as the mean percentage of patients colonized by *S. aureus* during 3 days preceding an acquisition. For the analysis of MSSA and MRSA acquisition only MSSA colonization pressure and MRSA colonization pressure were respectively assessed.

Antibiotic pressure.

Antibiotic pressure was evaluated by the total number of *defined daily doses* (DDD) administered to a patient during total stay [23].

Average DDD was calculated by dividing the total number of administered DDDs by the length of stay. The average number of DDDs administered per day per 1000 patient-days was calculated to compare antibiotic pressure between centers.

Cross-transmission.

Bacterial transmission was considered if a patient acquired an isolate, which was carried by another patient within two weeks preceding the acquisition. Isolates were considered genetically highly related if MLVA and *spa*-type were identical or if isolates differed only in a single locus in MLVA or had a highly related *spa* repeat sequence.

Statistical analyses.

All statistical analyses were performed using the Statistical Package for Social Studies (SPSS), version 12.0.2. Bivariate analyses were performed using Pearson's χ^2 cross-tabulation or student t-test (for independent samples). Center 1 was discarded in all analyses on MRSA acquisition as no MRSA was isolated in this center during the study period. Significance was set at $p \leq 0.05$.

Results

The total number of patients admitted to the participating ICUs in the study period was 629 (Table 1). Two hundred twenty four patients (36%) were colonized with *S. aureus* on at least one occasion during their stay on the ICU, of whom 49 (22%; range 0-46%) carried MRSA.

A total of 316 patients were not colonized by *S. aureus* on admission and remained on the ICU long enough to assess acquisition of *S. aureus* by at least one follow-up swab. Forty five (14% of at risk patients; range 0 - 20%) of these at risk patients acquired *S. aureus* (Table 1) during ICU stay, of whom 14 became colonized by MRSA (4% of at risk patients; range 0 - 12%). The remaining 271 patients were not colonized with *S. aureus* during ICU stay.

Variables considered for explaining the differences in acquisition were antibiotic pressure on the ICU prior to acquisition of *S. aureus*, length of stay (LOS) prior to acquisition of *S. aureus*, colonization pressure, beds per nurse ratio, hand disinfectant used per bed per day and ICU setting (single room treatment vs. open treatment).

The DDDs of antibiotics used on the ICUs ranged from 0.8 to 3.2 DDDs (Table 2). Patients who acquired MSSA or MRSA did not receive more antibiotics prior to acquisition than patients who did not acquire *S. aureus* ($p=0.30$ and $p=0.67$ for MSSA and MRSA resp.) (Table 3). No single antibiotic or antibiotic type was significantly correlated to acquisition of *S. aureus*.

Colonization pressure between the centers varied strongly. The average number of patients colonized ranged from 0.45 to 4.45 for MSSA and ranged from 0 to 2.05 for MRSA. Pre-acquisition colonization pressure was significantly higher for patients acquiring *S. aureus* (MSSA: $p=0.002$; MRSA: $p=0.005$) (Table 3). The average number of beds per nurse ranged from 1.5 to 2.6 beds per nurse and was significantly higher in patients acquiring both MSSA ($p=0.01$) and MRSA ($p=0.05$) than in patients remaining uncolonized throughout ICU stay (Table 3). The average number of hand disinfectant doses used per bed per day ranged from 27 to 97. The number of doses was not significantly related to MRSA acquisition. The number of doses was however significantly lower in patients acquiring a MSSA than patients remaining uncolonized ($p=0.02$).

Table 1. Prevalence and acquisition in participating ICUs.

Center	Prevalence			Acquisition		
		Pts + for *S. aureus*[1]			Acquired	
	Pts	*S. aureus*	MRSA	At risk pts[2]	*S. aureus*	MRSA
1	136	57 (42%)	0	44	8 (18%)	0
2	130	47 (36%)	9 (19%)	58	4 (7%)	0
3	115	52 (45%)	24 (46%)	57	9 (16%)	7 (12%)
4	151	51 (34%)	9 (18%)	91	18 (20%)	4 (4%)
5	64	12 (19%)	5 (42%)	46	6 (13%)	3 (7%)
6	33	5 (15%)	2 (40%)	20	0	0
Total	629	224 (36%)	49 (22%)	316	45 (14%)	14 (4%)

[1] Patients with a *S. aureus* positive culture at least once during ICU-stay.
[2] Patients with two or more follow-up culture swabs for assessment of acquisition.

Table 2. Participating centers

Center	Inclusion rate	ICU set-up[1]	Beds/nurse[2]	DDDs/day[3]	HD[4]
1	89%	2	1.5	1.4	34.4
2	81%	1	2.1	1.0	27.0
3	Unknown	2	2.0	0.9	90.6
4	96%	2	2.6	0.8	31.2
5	100%	2	2.4	1.6	46.3
6	100%	2	2.3	3.2	97.4
Average	93%		2.2	1.5	54.5

[1] Classification described in *Intensive Care information* in Methods
[2] Average number of beds per ICU nurse during a 24h period
[3] Average number of Defined Daily antibiotic Doses per patient per day
[4] Average number of hand disinfection doses bed per day

Patients who remained negative for *S. aureus* had a mean ICU stay of 10.3 days (range 8.0 - 12.9 days), whereas patients acquiring *S. aureus* on the ICU remained on the ICU for an average of 19.1 days (range 10.2 - 30.8 days). Patients admitted colonized by *S. aureus* remained on the ICU for an average of 10.7 days (range 7.4 - 19.4

Table 3. Bivariate analysis of variables associated with acquisition of *Staphylococcus aureus*.

Variables	Acquisition								
	S. aureus			MSSA			MRSA		
	yes	no	p	yes	no	p	yes	no	p
ICU set-up 1[1] ICU set-up 2	54 4	4 41	*0.05*	54 217	4 27	*0.25*	54 179	0 14	*0.03*
HD doses/ bed/ day[2]	48.2	56.5	*0.05*	48.2	59.8	*0.02*	48.2	49.3	*0.87*
Beds/ nurse	2.2	2.1	*0.03*	2.2	2.0	*0.01*	2.2	2.4	*0.05*
LOS prior to acquisition	10.0	7.9	*0.18*	10.0	8.3	*0.37*	10.0	6.9	*0.25*
Total LOS	10.0	19.1	*0.00*	10.0	18.7	*0.00*	10.0	20.0	*0.00*
Colonization pressure[3]	2.29	3.66	*0.00*	1.68	2.38	*0.00*	0.85	1.31	*0.00*
DDDs of antibiotics/day[4]	1.3	0.9	*0.28*	1.3	0.8	*0.30*	1.3	1.0	*0.67*

[1] ICU set-up as described in Methods
[2] Average number of handdesinfectant doses per bed per day
[3] Acquisition no = average number of patients colonized throughout study period. Acquisition yes = average number of colonized patients in three days preceding acquisitions
[4] During at risk period

days). The mean LOS prior to acquisition (LOS at risk) was 8.3 days for MSSA and 6.9 for MRSA. The difference in LOS (at risk) between patients who acquired and patients who did not acquire a *S. aureus* was significant for MSSA (p=0.001) and for MRSA (p=0.001).
Acquisition of MSSA was not lower in ICUs where all patients were treated in a single room (set-up 1) compared to ICUs where not all patients were treated in isolation (set-up 2) (Table 3). However, the number of MRSA acquisitions was significantly lower (p=0.03) in ICU set-up 1 compared to ICU set-up 2.

Genotyping.
All strains were genotyped (Table 4). The total number of *spa*-types found was 119. The total number of MLVA-types found was 237. Ten isolates were not typable by *spa*-typing. All strains were typable by **Table 4**. Genotyping and cross-transmission of *Staphylococcus aureus*

Center		Genotyping			Acquisitions			
	Isolates	***Spa***	**NT[1]**	**MTs[2]**	**Total**	**CT[3]**	**%**	**MRSA[4]**
1	97	22	0	55	8	5	63	0
2	120	36	1	62	3	1	25	0
3	85	13	0	22	9	6	67	6
4	105	35	2	78	18	4	22	1
5	25	9	7	15	6	2	33	1
6	12	4	0	5	0	0	0	0
Total	**444**	**119**	**10**	**237**	**45**	**18**	**40**	**8**

[1] Non typable isolates by *spa*-typing
[2] MLVA-types
[3] Possible cases of *Staphylococcus aureus* cross-transmission between patients (MRSA and MSSA) and in percentage of all acquisitions
[4] Cases of possible MRSA cross-transmission and in percentage of all cross-transmissions

MLVA typing. The total number of possible patient-to-patient cross-transmissions was 18. The percentage of acquisitions which could be related to patient-to-patient cross-transmission ranged from 22% to 67% between the participating centers. In center 6 no acquisitions and hence no cross-transmission occurred.

Cross-transmission possibly accounted for 40% of all *S. aureus* acquisitions on the ICUs studied. In 8 of these 18 cases the possibly cross-transmitted pathogen was MRSA (Table 5, appendix). In centers 1 and 2 no MRSA was transmitted. Six of the 8 cross-transmitted MRSA isolates were found in center 3. They were *spa*-types t032, t1474 and t2577 (both the latter genetically related to t032) and closely related in MLVA-type. In center 4 one MRSA was possibly cross-transmitted, which was not typable by *spa*-typing. However, it was identical in MLVA-type to a MRSA found in a patient admitted the same day, which was also not typable by *spa*-typing. The cross-transmitted MRSA isolate in center 5 was also not typable by *spa*-typing; transmission assessment was based on MLVA- typing.

The transmitted MRSA isolates from different centers were not similar in MLVA-type.

In centers 1 and 2 no MRSA isolates were acquired. The cross-transmitted MSSA isolates were spa-types t002, t1889 and t015 (unrelated *spa*-types) in center 1. The isolates were not related in MLVA-type. In center 2 acquired isolates were t068, t012 and a non-typable isolate. The acquired t068 isolate cross-transmitted to another patient. In center 4 cross-transmitted MSSA isolates were *spa*-types t012, t238 and t084. One t084 isolate was acquired and cross-transmitted to another patient. In center five a t645 MSSA isolate was acquired and cross-transmitted to another patient.

Discussion

Considerable differences have been reported on rates of acquisition and transmission of *S. aureus* on the ICU and the efficacy of intervention measures [16,41]. The contribution of variations in sampling and culturing methods to these differences is not clear. In this study we have assessed the acquisition of *S. aureus* in different European ICUs using a uniform study protocol to enable a reliable comparison between different ICUs.

Antibiotic pressure has been described as a risk factor for the acquisition of MRSA [9,10] and use of antibiotics during and prior to ICU admission has been shown to increase the number of MRSA infections [12,13,28]. Our results do not show an increased risk of acquiring *S. aureus* colonization if antibiotics are administered during ICU admission even though only the "at risk" period was taken in account. We could not show one or more individual types of antibiotics to be strongly related to the increased risk of acquisition. Unfortunately, the information on the administered antimicrobial agents to the patients remaining negative for *S. aureus* colonization throughout the study period was not obtained for two centers and could therefore not be evaluated in our results.

It is generally accepted that LOS on an ICU is correlated with acquisition of MRSA. However, Grundmann et al did not show an increased LOS before of acquisition for MRSA [29]. This is in agreement with our results since we also did not find a correlation between LOS prior to acquisition and the acquisition of *S. aureus*. Remarkably, patients who were already colonized with *S. aureus* when admitted to the ICU had a total LOS of 10.7 days, which was comparable to the total LOS for patients who did not become colonized (10.3 days). However, patients acquiring MSSA or MRSA during their ICU stay had a total LOS of 18.7 and 20.0 days respectively. This could be explained by the fact that patients with a poorer health status (APACHE II and SAPS II scores) and thus a

longer LOS are at greater risk of acquiring a *S. aureus*, as previously shown for MRSA [8,18,29]. The average LOS described in our results is relatively high. This can be explained by the fact, that patients not having at least one follow-up swab taken in order to assess acquisition were excluded from this evaluation.

Colonization pressure was shown to be strongly related to the acquisition of MRSA [8] and shown not to be related in other studies [15,27]. In our study colonization pressure within an ICU significantly correlated with a higher acquisition rate of both MSSA and MRSA. The variability of these results on colonization pressure may be explained by the difference in methods used for calculating "colonization pressure".

A significant correlation was found between the number of beds per nurse and the acquisition of MSSA and MRSA. Dancer et al. reports on an increase in acquisitions of MRSA during a deficit of trained nurses on the ICU [27] and general nurse workload is related to the number of infections and mortality of MRSA [26].

To obtain an estimate of the level of hand hygiene on the different ICUs the number of doses of hand disinfectant used per bed per day was assessed. Individual hand disinfection compliance was not investigated further, as this may lead to questionable results due to lack of objective measurements and the Hawthorn effect [42]. The amount of hand disinfectant used per bed per day varied by a factor of 3.6 and this correlated to a higher number of MSSA acquisitions. However, the amount of hand disinfectant used was not significantly related to MRSA acquisition. The wide variation in use of hand disinfectant found between the different ICUs cannot be explained and is clearly a possible deficiency of the used method.

A lower acquisition rate for MRSA through isolation treatment of colonized patients has been postulated in several studies [43-47]. Nevertheless, a meta-analysis did not show indisputable evidence that isolation treatment of colonized patients leads to a reduced level of MRSA acquisition [16]. Our results show that acquisition of MRSA

was lowest on an ICU where all patients were treated in single rooms. This result was not shown for MSSA. Our result is in agreement with a study which showed that no acquisition of MRSA and two cases of MSSA acquisition occurred in a single room ICU setting [31].

The total number of possible cross-transmissions was comparable to the results found in similar studies [23,24]. However, these studies assessed MRSA only. In our results the patient-to-patient transmission of MRSA is relatively low. In addition, six out of 8 these possible MRSA transmissions were found in 1 center, which is endemic for an EMRSA-15, *spa*-type 032 clone [48]. This clone was found in all possible cross-transmissions, probably leading to an overestimation of the number of MRSA cross-transmissions. No outbreak situation was seen during the study period, as the number of MRSA carriers found did not differ from the number described previously [48]. The number of cross-transmissions of MSSA isolates was comparable to MRSA. No one *spa*-type was found to be predominant in MSSA cross-transmissions, although in center 1 *spa*-type t002 was found in 3 out of 5 cross-transmitted isolates. As these isolates were not related in MLVA-type, this cannot be interpreted as an outbreak strain, demonstrating the relevance of MLVA-typing next to *spa*-typing in a clinical situation.

The present study has a number of limitations. The most important is the relatively short period of inclusion per center and the therefore low numbers of acquisition. This has unfortunately led to a questionable multivariate analysis due to underpowering. Additionally, a number of patient characteristics was not taken in account. Confounders in this study could be the "average health status" of patients on the different ICUs. The number of surgical and ventilated patients could also influence the acquisition rate on an ICU [18,49].

Summarizing, the impact of length of stay prior to acquisition of *S. aureus* and antibiotic pressure prior to acquisition of *S. aureus* on ICU acquisition could not be demonstrated. Colonization pressure, the

number of beds per nurse and treatment of all patients in single rooms correlate to the number of acquisitions on an ICU. Hand disinfectant use was correlated to MSSA acquisition, but not to MRSA acquisition. Patient-to-patient cross-transmission was comparable between MSSA and MRSA.

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Patients colonized by multiple *Staphylococcus aureus* strains in European Intensive Care Units

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Introduction

Patients and healthy individuals intermittently and inconsistently carry different MRSA subtypes [1-3]. In the present study we assessed clonality of both MSSA and MRSA in individual patients admitted to 6 ICUs using *spa*-typing and MultiLocus Variable number of tandem repeat Analysis (MLVA) as an addition to our previously published study, to which we refer for further information on patients, ICUs and genetic characteristics of isolated *S. aureus* strains [4].

Methods

The study was undertaken in 6 European university and teaching hospitals during a period of 3 months per center. Nasal swabs were taken from all patients within 48h of admission and twice a week. Swabs were cultured for 24h at 37°C in 4 ml trypsin soy broth with 5 mg/L aztreonam and subsequently plated on mannitol salt agar and blood agar plates and incubated for 48h at 37°C.

All *S. aureus* strains were examined by a triplex PCR for 16S rRNA, *nuc* and *mecA* genes [5]. DNA was isolated using a Nucleospin Tissue Kit (Machinery-Nagel) according to manufacturer's protocol.

All *S. aureus* isolates were genotyped. MLVA was performed by PCR of 6 variable number tandem repeats, shown previously to be at least as discriminatory as PFGE for typing of *S. aureus* [6]. *Spa*-typing was performed using 5'-ACGAGTAGTGCCCTTTGCTT and 3'-GCTCAAGCACCAAAAGAGGA primers (Invitrogen, the Netherlands) or 5'-TAAAGACGATCCTTCGGTGAGC and 3'-CAGCAGTAGTGCCGTTTGCTT primers (Isogen Life Sciences, the Netherlands). DNA sequencing was performed using the chain termination method (Baseclear BV, Leiden, the Netherlands). Bionumerics (Applied Maths, Belgium) was used to analyze obtained sequences and assign *spa*-types. Novel *spa*-types were submitted online to the Ridom database (www.SpaServer.ridom.de).

Isolates were considered to be different *S. aureus* strains when they differed in more than 2 loci in MLVA and/or differed in *spa*-type. Isolates with an identical or closely related MLVA-type (≤ 1 single locus difference) and identical *spa*-type were considered to be identical. Isolates containing a double locus difference in MLVA-type and/or an identical or closely related *spa*-type were considered to be closely related. In case a strain was not typable by *spa*-typing, only MLVA was assessed to determine relatedness.

Results

A total of 371 of all 629 patients (52%) had more than one culture taken and 224 (36%) had one or more *S. aureus* positive cultures during ICU stay, of which 49 (8% of total) were carrier of MRSA. In total, 952 swabs were collected from the 224 *S. aureus* carriers, of which 425 (45%) were *S. aureus* positive.
In these isolates 119 *spa*-types were found. Ten isolates were not typable by *spa*-typing. All strains were typable by MLVA-typing. In total, 237 MLVA-types were found.
From 87 patients multiple positive *S. aureus* culture swabs were obtained. The remaining 137 patients had only one positive swab. Out of the 629 included patients 35 (6%) carried either 1 strain or ≥2 genetically closely related isolates and 35 (6%) carried 2 genetically distinct strains. Two of these last mentioned were possible cases of cross-transmission, as a patient carrying a genetically identical *S. aureus* isolate was admitted to the ICU in the two weeks prior to the acquisition. The remaining 17 (3%) carried more than 2 strains during ICU stay. Five of these were possible cross-transmissions. No patients carried both closely related isolates and different strains. Seven patients (1% of total) carried MSSA or MRSA (with different *spa*- and MLVA types) on different days of culturing.

Table 1. Carriage of multiple strains

Center	Pts[1]	Carriers[2]	Number of strains carried[3]				
			1	**CR[4]**	**2**	**>2**	**Total**
1	136	57 (42%)	2	2	6	6	**16**
2	130	47 (36%)	9	0	14	3	**26**
3	115	52 (45%)	9	4	3	0	**16**
4	151	51 (34%)	1	2	10	7	**20**
5	64	12 (19%)	4	1	1	1	**7**
6	33	5 (15%)	1	0	1	0	**2**
Total[5]	**629**	**224 (36%)**	**26 (4%)**	**9 (1%)**	**35 (6%)**	**17 (3%)**	**87**

[1] All patients included in the study
[2] Patients with at least one *S. aureus* positive culture during ICU stay
[3] Number of strains carried by patients who had multiple positive *S. aureus* cultures during ICU stay
[4] Patients carrying closely related (CR) strains
[5] Percentages of all included patients

Discussion

It has long been believed that patients predominantly carry one distinct *Staphylococcus aureus* strain. A number of contemporary studies have shown clonal diversity in *S. aureus* colonization [1-3]. Vandenbergh et al. studied persistent carriership among patients and concluded that only a fraction will carry a genetically identical strain over years [3]. Simultaneous carriage of clonally distinct MSSA and MRSA strains was shown in a subgroup of (previously) hospitalized persons [2]. Lim et al. reported that ICU patients carry clonally diverse MRSA strains during their ICU stay [1].

In total 52 (8%) patients carried 2 or more distinct *S. aureus* strains during ICU stay. This is comparable with the results reported by Maslow et al. [2]. Seven (1%) patients carried both MRSA and MSSA during ICU stay. The question is, whether these different strains are endogenous or acquired isolates. Acquisition of an exogenous isolate (by patient-to-patient cross-transmission) was possibly the case in 7 out of the 52 cases of multiple strain carriage in the present study. In 5 of these cases the patient was carrier of 3 or more distinct *S. aureus* strains overtime. In the remaining 45 cases cross-transmission could not be shown. As the ICU acquisition of *S. aureus* is related to antibiotic use, which is usually high on ICUs [7,8] and to poor health status [9,10], an exogenous source would be conceivable. However, our results do not strongly support this hypothesis. In the case of endogenous strains, the question arises, whether the strains are simultaneously and thus polyclonally colonizing the nasal tissue. Unfortunately, we did not analyze multiple colonies from positive swabs to ascertain polyclonal carriage.

In the present study only 45% of swabs obtained from colonized patients were positive for *S. aureus*. This finding can be explained in two ways. First, patients could lose nasal colonization and be recolonized during ICU stay. Secondly, the method used for determining nasal colonization status, may lack analytical sensitivity. Lim et al. report a low concordance between two culture swabs taken on the same day. In almost 60% of these nasal cultures one culture was negative and the other positive [1].

The clinical implications of our findings are apparent. Patients receiving treatment for an *S. aureus* infection could become reinfected with another *S. aureus* strain, which the patient could be carrying simultaneously. In addition, the possible lack of sensitivity of

nasal culture swabs may result in an underestimation of MRSA colonization. This could lead to a higher spread of MRSA and an increased number of nosocomial infections.

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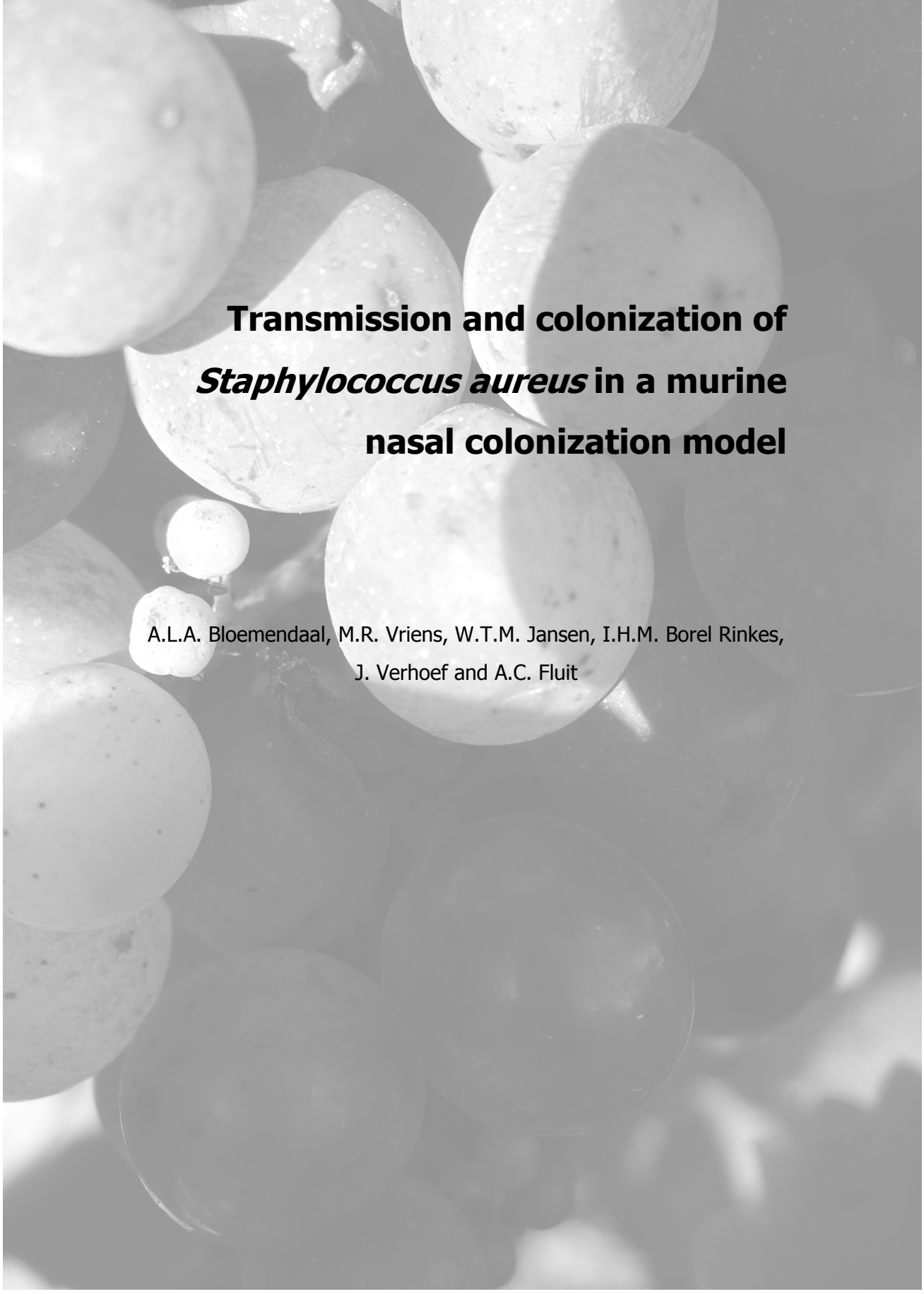

Transmission and colonization of *Staphylococcus aureus* in a murine nasal colonization model

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Abstract

Nasal colonization by *Staphylococcus aureus* is an important risk factor for the development of a nosocomial infection. Acquisition of nasal colonization by *S. aureus* increases mortality in hospitalized patients, but little is known about the transmission dynamics of *S. aureus*. To study *S. aureus* transmission, colonization and colonization persistence we developed a murine transmission model. In 20 cages 2 out of 10 mice were nasally inoculated (at 5×10^8 CFU per mouse) with either MSSA (10 cages) or MRSA (10 cages). On days 5, 15, 25 and 40 all mice in a cage were swabbed or sacrificed and nasal colonization and colony forming units were determined in all 10 mice by nasal dissection or by nasal swab. Spread and subsequent stable colonization by both MSSA and MRSA from colonized to uncolonized mice within a cage was seen. This experiment was repeated using nasal swabs to determine colonization. At day 5, an increased number of colonized mice was observed in the MSSA group compared to the MRSA group ($p = 0.003$). On day 40, the average number of CFUs per mouse was higher for MRSA than for MSSA ($p=0.06$). Fecal-oral transmission was shown to be a possibly important transmission route in this model. These results suggest a more rapid spread of MSSA compared to MRSA. However, MRSA does show a more stable nasal colonization after a longer period of time.

Introduction

Staphylococcus aureus is a major pathogen in nosocomial and community acquired infections. Approximately 30% of healthy individuals carry *S. aureus* on the skin and/or mucous membranes. A positive carrier status significantly increases the chance of acquiring a nosocomial infection, predominantly of wounds, catheters and intravascular devices [1]. In an ICU setting approximately 30% of acquired infections are caused by *Staphylococcus aureus* [2]. Mortality rates are significantly increased in patients carrying MRSA; overall ICU mortality was 18% in a French study, mortality increased to 45% in MRSA colonized patients and to 58% in MRSA infected patients [3].
Little is known about the dynamics of transmission of *Staphylococcus aureus* in an ICU setting. In our hospital MRSA was shown to be more epidemic than methicillin-susceptible *S. aureus* (MSSA) in a Dutch intensive care unit [4]. In a European study, which assessed the spread of *S. aureus* on different ICUs, no difference in epidemicity could be shown between MRSA and MSSA [5].

In the present study we assess transmission, colonization and colonization persistence of *Staphylococcus aureus* in a murine nasal colonization model. To answer the question whether MRSA is more contagious than MSSA, the possible differences in transmission and colonization dynamics between a genetically identical strain set of MSSA and MRSA. The whole genome of these isolates was obtained, showing no genetic difference, except for a type IV SCC*mec* [6]. These strains were previously described by Wielders *et al.* [7].

Methods

Animals

The mice used for this study were 6 week old female Balb/c's, obtained from Harlan (Horst, the Netherlands) and Charles River (Boston, USA). The animals were kept in a separate room in the Infections Unit of the General Animal Laboratory of the University of Utrecht. The mice were housed per 10 in filtertop cages and were fed water and food *ad libitum*. The study was performed with permission of the local Animal Ethics Committee (DEC).

Staphylococcus aureus strains and growth conditions

The *S. aureus* strains used for the experiment were the MSSA WKZ-1 and MRSA WKZ-2 (type IV SCC*mec*), previously described by Wielders *et al.* [7] and Jansen *et al.* [8]. Recent whole genome sequencing has shown that, apart from the type-IV SCC*mec* in WKZ-2, no differences including single nucleotide polymorphisms were found. Therefore, WKZ-1 and WKZ-2 are a fully isogenic MRSA-MSSA pair [6]. The strains were stored at -80°C. Strains were grown on 5% sheep blood agar for 24 hours at 37°C. Bacteria were suspended in saline and quantified by optical density measurement set at OD_{660} = 1 (1.3 x 10^9 cells/ml). To confirm a comparable CFU count WKZ-1 and WKZ-2 were plated by a serial dilution on 5% sheep blood agar plates.

Transmission experiments

The study was performed in two different experimental setups. In the first experiment the same mice were swabbed nasally on sample days to assess colonization (4 cages total). In the second setup the mice were euthanized and the nasal area was dissected to assess colonization (16 cages total).

Ten cages with 10 mice each were assigned to each bacterial strain. In each of the 10 MSSA cages, 2 mice were inoculated with 5 x 10^8 CFU MSSA WKZ-1 in 20 µl saline. In each of the 10 MRSA cages, 2 mice were inoculated with 5 x 10^8 CFU MRSA WKZ-2 in 20 µl saline. Inoculation was performed using a sterile pipette. The inoculated mice were marked by an ear tag.

On sample days (days 5, 15, 25 and 40 post-inoculation) all mice in 2 MSSA cages and all mice in 2 MRSA cages were swabbed nasally using a sterile loop. The tips of the loops were cut off with sterile

scissors and transferred into 200 µl saline. After rigorous vortexing, 10 µl was plated on mannitol salt agar (MSA) plates and incubated for 48 h at 37°C. On the same sample days all mice in 2 MSSA and all mice in 2 MRSA cages were euthanized by cervical dislocation. The external nasal region was cleaned with 80% alcohol. Nasal tissue was excised with sterile scissors and dissected in 2 equal parts. One part was grown for 24 h in 4 ml trypsin soy broth (TSB) with 5 mg/l aztreonam (Squibb, U.S.A.) at 37°C. After rigorous vortexing 10 µl was plated on MSA plates and incubated for 48h at 37°C.
The other part was grinded in 100 µl saline. The tissue suspension was centrifuged at 4000 rpm for 10 min. The supernatant was plated on 5% sheep blood agar plates in a dilution series to obtain a nasal CFU count.

Feces colonization

Feces samples were taken from all mice at sacrifice to assess *S. aureus* colonization of the digestive tract. Feces samples were suspended in 4 ml saline and 100 µl suspension was pipetted in 4 ml trypsin soy broth with 5 mg/l aztreonam and incubated for 24h at 37°C. After vortexing 10 µl broth was plated on MSA plates and incubated for 48h at 37°C.

Feces-oral transmission

To assess the relevance of feces colonization in the transmission of *S. aureus* in a murine model, feces was obtained from the MSSA and MRSA cages and introduced in "clean cages" holding 6 non-inoculated mice. Feces was freshly obtained and taken from the cage floor. Freshly obtained droppings were dissected to retrospectively assess *S. aureus* colonization of introduced feces.

Strain verification

Presence of *Staphylococcus aureus* was confirmed by DNase test and coagulase test in all experiments. To confirm strain identity isolates were typed by MultiLocus Variable Number of Tandem Repeats Analysis (MLVA), as described by Ikawaty *et al.* [9].

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 12.0.1. Categorical variables were compared using the Chi-square test and continuous variables using the Mann-Whitney U-

test. Continuous variables were calculated by paired T-test. Significance was set at $p < 0.05$.

Results

Inoculation and spread

Using two different sampling methods to assess the nasal carriage of MSSA and MRSA in mice, transmission of both bacterial strains from inoculated mice to non-inoculated mice was observed (Table 1).

In the combined results (Fig. 1) MSSA showed a mean number of colonized mice of 7.8 per cage on day 5, whereas MRSA had colonized 4.3 mice ($p = 0.00$). On day 15, the mean colonization number of colonized mice by MSSA and MRSA was 9.8 and 8.5 mice respectively ($p = 0.02$), whereas on day 25 these were 7.3 and 9.0 mice, respectively ($p = 0.01$) and on day 40 4.0 and 5.3 mice were colonized, respectively (n.s.).

The number of CFU per strain on the days post-inoculation can be seen in Figure 2. A near significant difference ($p = 0.06$) was seen on day 40.

All but one inoculated mice remained positive for *S. aureus* colonization throughout the duration of the experiments. On day 40 one inoculated mouse was found to be negative. All animals survived until the day of sacrifice. No complications were observed.

Feces colonization

Simultaneous colonization of nares and feces was seen in all but six cases. In two cases in the MRSA group 2 mice were found negative for nasal colonization, but positive for fecal colonization on day 5. In four cases a mouse was found positive for nasal colonization but negative for fecal colonization. This was the case in one mouse on day 5. Three mice showed the same pattern on day 15.

Table 1. Number of mice colonized by MSSA and MRSA on different days for both experimental methods separately and cumulatively.

Days[1]	MSSA			MRSA		
	swab (n=20)	dissection (n=20)	total (n=40)	swab (n=20)	dissection (n=20)	total (n=40)
5	16	15	31	10	7	17
15	20	19	39	20	14	34
25	16	13	29	20	16	36
40	4	12	16	10	11	21

[1]days post-inoculation

Feces-oral transmission

All freshly obtained droppings from nasally colonized mice, which were introduced in the clean cages, were positive for *S. aureus*. All mice were positive for colonization by *S. aureus* in case of MSSA and MRSA introduction.

Strain verification

All collected *S. aureus* strains had identical MLVA types.

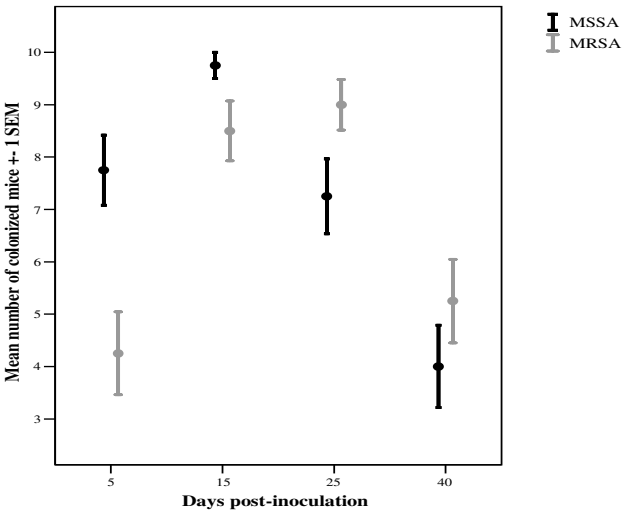

Fig 1. The mean number of mice colonized per 10 mouse cage by MSSA and MRSA. Mice were inoculated with 5 x 10^8 CFU MSSA (black bars) and MRSA (grey bars). Samples were taken on days 5, 15, 25 and 40 post-inoculation.

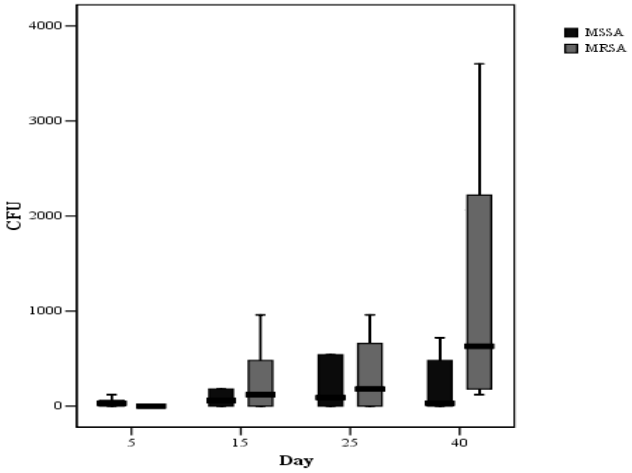

Fig. 2. Number of colony forming units for MSSA (black bars) and MRSA (grey bars) groups on days 5, 15, 25 and 40 in a box plot (median and range).

Discussion

Many studies have been undertaken to assess virulence and resistance mechanisms in *Staphylococcus aureus*. There is no conclusive evidence for an increased or decreased virulence of MRSA compared to MSSA [10,11], although a meta-analysis does show an increase in mortality in MRSA bacteremia compared to MSSA bacteraemia [12]. No definite explanation for this increase can be provided, but a delay in appropriate treatment, due to underestimation of MRSA in a non-outbreak situation is a likely reason.

One may presume that the epidemicity (transmission, colonization and colonization persistence) of MRSA should not differ from that of MSSA, as both strains are essentially identical, apart from SCC*mec*. A number of studies has been performed on fitness cost of resistance acquisition by different pathogens. Ender *et al.* showed a reduced growth rate after integration of a type I SCC*mec* in an MSSA in a direct competition growth experiment [13]. Lee *et al.* showed a fitness cost after type I SCC*mec* incorporation, but not after type IV SCC*mec* incorporation. This was assessed by a glucose uptake and a growth rate experiment [14]. Karauzum *et al.* showed a decreased fitness in a ST8 MRSA compared to a ST8 MSSA, but not in a ST5 MRSA and MSSA (both type IV SCC*mec*) in an adhesion model and a murine sepsis model [15]. A decrease in fitness was also seen after acquisition of resistance to other antimicrobials [16-18]. In theory, the presence of the SCC*mec* cassette may result in an increased or decreased epidemicity. This effect may be achieved through polar effects due to the insertion of SCC*mec* into *orfX* on the staphylococcal chromosome. Genes on the SCC*mec* may influence regulatory peptides through an unknown mechanism as shown by Kaito *et al.* [19]. They describe the role of the *fudoh* gene in the ability of *S. aureus* to spread on agar and to infect mice. The presence of *fudoh* decreased the spread rate of both MSSA and MRSA. In addition, in a

murine infection model, *fudoh*-positive *S. aureus* isolates were less virulent. A microarray showed that this gene also affects the expression of a number of other genes, which are not located on SCC*mec*. Function and location of these genes are not stated. The *fudoh* gene has been found on type II and III SCC*mec*. It was not shown to be present on type I, IV and V SCC*mec*.

In the present study we have developed a murine epidemicity model, to study the transmission and colonization persistence of *Staphylococcus aureus*. To this end we used a MSSA and a MRSA strain (WKZ-1 and WKZ-2, respectively), which are genetically identical, except for a type IV SCC*mec* cassette in the WKZ-2 strain. As a basis for our method we used the murine nasal colonization model as described by Kiser *et al.* [20], which showed a stable colonization of the murine nares with *Staphylococcus aureus* after inoculation with at least 10^8 CFU.

The results of our study also show a stable colonization of all inoculated mice for 40 days, after inoculation with 5 x 10^8 CFU in correlation with the finding of Kiser *et al*. Furthermore, our results show a successful spread of both MSSA and MRSA from inoculated mice to non-inoculated mice within a cage. This result was found in 2 different experimental set-ups and is therefore considered reproducible. The newly colonized mice showed a stable colonization for at least 15 days and in almost half of the cases the mice were still colonized by *S. aureus* on day 40.

A difference was found in the rate of transmission between MSSA and MRSA. MRSA was seen to spread less rapid than MSSA. However, there was no significant difference in number of colonized mice after 40 days. On the contrary to the number of mice colonized, the number of colony forming units (CFU) was found to be higher in MRSA than MSSA on day 40, suggesting a more stable colonization after a longer period of time. This may suggest an intrinsic factor on the type-IV SCC*mec* on the WKZ-2 chromosome influencing colonization persistence, but further experiments should be

undertaken to assess this suggestion. Sequence analysis of the SCC*mec* element did not yield an obvious gene candidate that may explain the difference.

To assess the relevance of digestive tract colonization and coprophagia in a murine bacterial epidemicity model, we studied fecal colonization and fecal-oral transmission by introducing colonized feces in cages holding non *S. aureus* colonized mice. A major limitation in this experiment was the impossibility of excluding the role of environmental contamination by the feces, due to ethical restrictions (i.e. cages without ground covering). Therefore it is not possible to conclusively state, that the positive colonization of all clean mice is attributable to the eating of the feces. It does however show the relevance of digestive tract colonization in the transmission of *S. aureus* in a murine model. Furthermore, the experiment shows a clear correlation between nasal colonization and digestive tract colonization by *S. aureus*. The few cases of negative nasal carriage and positive digestive tract *S. aureus* carriage could be due to non-detectable colonization rates in the murine nares in the early stages of colonization.

We believe we have demonstrated the possibility of using a murine nasal colonization-transmission model for the assessment of epidemicity of *Staphylococcus aureus* strains. We have shown a stable colonization and spread of both MSSA and MRSA. The differences seen in the epidemicity of the genetically highly related WKZ-1 and WKZ-2 strains should be further investigated, following the suggestion of influence on transmission by the introduction of the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*) in *Staphylococcus aureus*.

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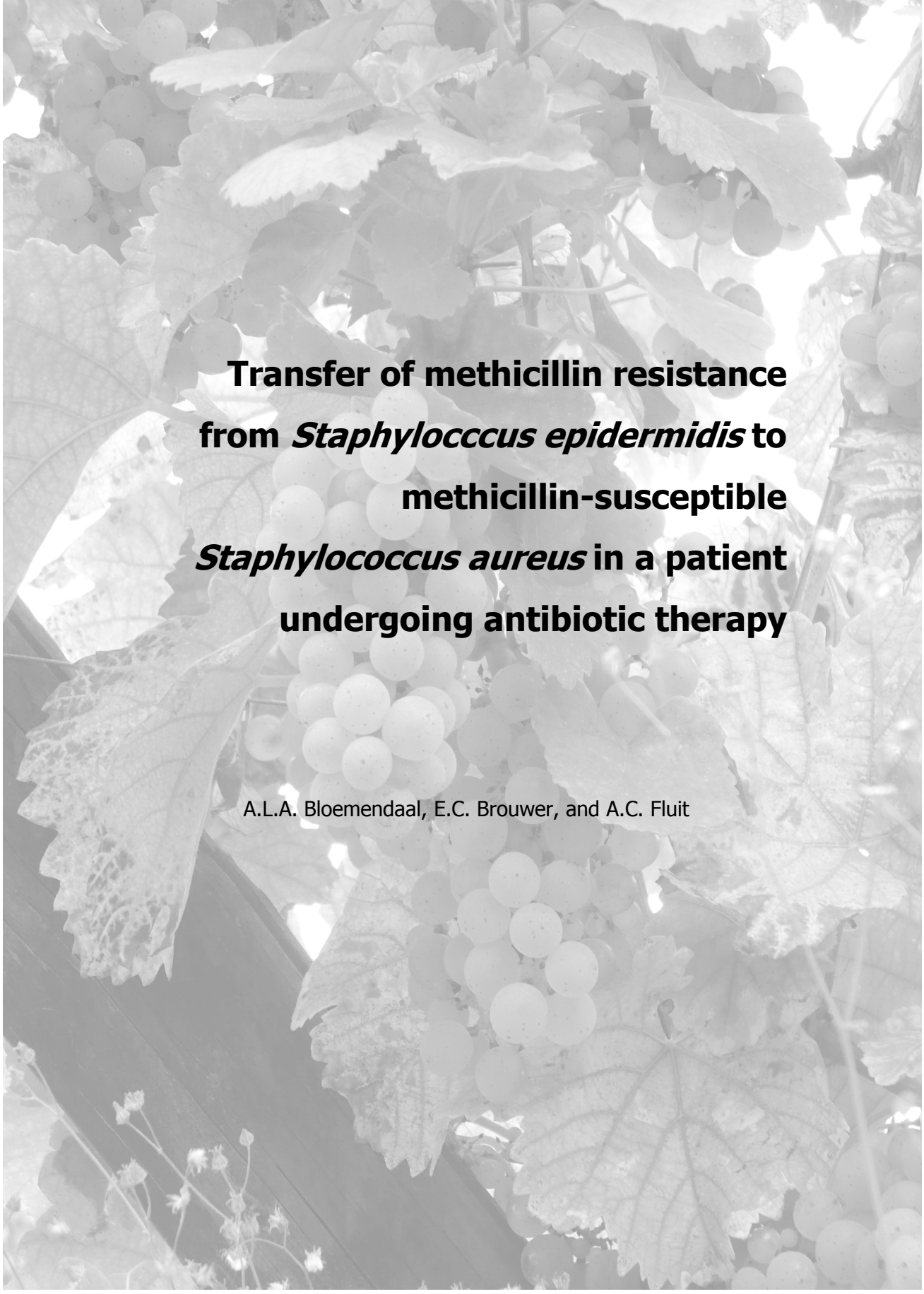

Transfer of methicillin resistance from *Staphylocccus epidermidis* to methicillin-susceptible *Staphylococcus aureus* in a patient undergoing antibiotic therapy

A.L.A. Bloemendaal, E.C. Brouwer, and A.C. Fluit

Abstract

Background. The *mecA* gene, encoding methicillin resistance in staphylococci, is located on a mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*). Horizontal, interspecies transfer of this element could be an important factor in the dissemination of methicillin-resistant *S. aureus* (MRSA). Previously, we reported the isolation of a closely related methicillin-susceptible *Staphylococcus aureus* (MSSA), MRSA and potential SCC*mec* donor *Staphylococcus epidermidis* isolate from the same patient. Based on fingerprint techniques we hypothesized that the *S. epidermidis* had transferred SCC*mec* to the MSSA to become MRSA. The aim of this study was to show that these isolates form an isogenic pair and that interspecies horizontal SCC*mec* transfer occurred.

Methods. Whole genome sequencing of both isolates was performed and for the MSSA gaps were closed by conventional sequencing. The SCC*mec* of the *S. epidermidis* was also sequenced by conventional methods.

Results. The results show no difference in nucleotide sequence between the two isolates except for the presence of SCC*mec* in the MRSA. The SCC*mec* of the *S. epidermidis* and the MRSA are identical except for a single nucleotide in the *ccrB* gene which results in a valine to alanine substitution. The main difference with the closely related EMRSA-16 is the presence of SaPI2 encoding toxic shock syndrome toxin and exfoliative toxin A in the MSSA-MRSA pair. No transfer of SCC*mec* from the *S. epidermidis* to the MSSA could be demonstrated *in vitro*.

Conclusion. The MSSA and MRSA form an isogenic pair except for SCC*mec*. This strongly supports our hypothesis that the MRSA was derived from the MSSA by interspecies horizontal transfer of SCC*mec* from *S. epidermidis* O7.1.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community-acquired infections. Resistance to methicillin is a result of the expression of penicillin-binding protein 2a (PBP2a), which causes a low affinity for ß-lactam antibiotics. PBP2a is encoded by the *mecA* gene, which is located on a mobile genetic element called Staphylococcal Cassette Chromosome *mec* (SCC*mec*) [1]. To date, six types of SCC*mec* have been described [2,3], and recently two novel SCC*mec* types have been reported, tentatively named types VII and VIII [4,5].

Horizontal transfer of mobile genetic elements contributes to the worldwide dissemination (multi)-resistant pathogens. Leverstein-van Hall *et al.* described a high rate of interspecies transfer of resistance genes among multidrug resistant Enterobacteriaceae in patients [6]. Berglund *et al.* described the likely transfer of a type V SCC*mec* from methicillin resistant *Staphylococcus haemolyticus* to *S. aureus* in a neonatal intensive care unit [7]. Although this interspecies transfer of SCC*mec* could contribute profoundly to the dissemination of MRSA, evidence is still inconclusive.

Previously, we described the isolation of a successive pair of *mecA*$^-$ and *mecA*$^+$ *S. aureus* and a methicillin resistant *Staphylococcus epidermidis* isolate, which was likely the donor of the SCC*mec*, from a male infant suffering Pierre Robin syndrome. The patient was intubated and mechanically ventilated 4 days after birth because of respiratory insufficiency. A suspected respiratory tract infection was treated with amoxicillin/clavulanic acid. As he became bacteraemic after 3 days the antibiotic regimen was changed to amoxicillin and cefotaxime. When his blood cultures grew *mecA*$^-$ methicillin-susceptible *S. aureus* (MSSA) treatment was changed to flucloxacillin. He seemed to recover, but amoxicillin/clavulanic acid treatment was reinstated for 10 more days on day 32 after the respiratory tract infection had recurred. On day 56, routine cultures from nasal swabs unexpectedly showed a *mecA*$^+$ MRSA. Several strains of *mecA*$^+$ coagulase-negative staphylococci (CNS) were also identified. Pulsed-Field Gel Electrophoresis (PFGE) of the isolates using *Sma*I showed a 40 kb shift of a single DNA fragment between the MSSA and MRSA. Southern hybridization showed that the larger DNA fragment contained the *mecA* gene. The MRSA and MSSA had identical ribotypes and a rare phage type not recorded previously by the Dutch

reference center. The PFGE pattern was also not encountered among the profiles of 312 European MRSA isolates. The isolates were susceptible for all antibiotics tested except that the MRSA was resistant to ß-lactam antibiotics. Southern blotting of MRSA and CNS DNA cut with *Cla*I, *Eco*R1, and *Hind*III using a SCC*mec*-specific probe obtained from the MRSA showed identical fingerprints for the MRSA and one of the CNS isolates. From these data we concluded that likely transfer of SCC*mec* from a *S. epidermidis* to a MSSA had occurred [8].

To obtain more definite proof that the *S. epidermidis* was the donor of the SCC*mec*, the whole genome sequences of the MSSA and MRSA were determined as well as the sequence of the SCC*mec* sequence of the *S. epidermidis* isolate.

Materials and Methods

DNA sequencing

The MSSA and MRSA isolates WKZ-1 and WKZ-2 were sequenced by KeyGene (Wageningen, The Netherlands) using 454 sequencing technology. For WKZ-1 the gaps between the contigs with the exception of the rRNA operons were closed by PCR amplification of the missing fragments followed by conventional DNA sequencing. MRSA252 was used as a scaffold. Annotation was performed by JCVI. The sequencing of WKZ-1 has been reported in GenBank under project ID 40253. The SCC*mec* sequence is accessible under no. GQ918137.

Single-nucleotide polymorphism (SNP) analysis was performed using NUCMER (http://mummer.sourceforge.net) followed by confirmation using PCR amplification and conventional DNA sequencing.

The SCC*mec* of the *S. epidermidis* was sequenced by conventional sequencing of PCR products. PCR amplifications were based on the sequence of the SCC*mec* of WKZ-2. Conventional sequencing was performed by BaseClear (Leiden, The Netherlands).

In vitro SCC*mec* transfer

To select *S. aureus* that may have obtained SCC*mec* from the *S. epidermidis* donor strains during *in vitro* mating experiments the MSSA was made resistant to linezolid by serial transfer. Bacteria were mated on nitrocellulose filters. Mating experiments without antibiotics were performed using TSA 5% sheep blood plates (BD Diagnostic Systems, USA). Mating experiments under antibiotic pressure were performed using cloxacillin at concentrations of 0.5, 0.25 and 0.125 µg/mL and cefotaxime at concentrations of 1.0, 0.5, 0.25 µg/mL in LB agar. Conjugants were selected using both linezolid and cloxacillin at 4.0 µg/mL each.

Antibiograms

To assess antimicrobial resistance in the WKZ-1 MSSA and the *S. epidermidis* 07.1 isolates, the minimal inhibitory concentration (MIC) were obtained for a number of clinically relevant antibiotics. Results are shown in Table 1.

Results

For MSSA WKZ-1 a total of 45 contigs larger than 1000 nucleotides was obtained with an average read size of 244 nucleotides and a 23.5-fold coverage, whereas for WKZ-2 a total of 136 contigs larger than 1000 nucleotides were obtained with an average read size of 187 nucleotides and a 9.9-fold coverage. The isolates belong to Sequence Type (ST) 30. MRSA252, which is an EMRSA-16 and has ST36 and belongs to the same Clonal Complex (CC30) was the most closely related strain from which the whole genome was sequenced. Therefore, the sequence of this strain was used as a scaffold to determine the order and orientation of the contigs for further DNA sequencing of the gaps between the contigs. Except for SCC*mec* no difference in the mobile genetic elements of WKZ-1 and WKZ-2 was found.

The WKZ isolates differ from MRSA252 in a number of mobile genetic elements. The WKZ-2 MRSA contains a SCC*mec* type IV, whereas MRSA252 carries a SCC*mec* type II of 58.8 kb which includes pUB110 encoding bleomycin and kanamycin resistance and Tn*554* encoding resistance to erythromycin and streptomycin. At the position of the *Staphylococcus aureus* Pathogenicity Island 4 (SaPI4) in MRSA252 the WKZ isolates carries a slightly different SaPI. It shows a difference in 6 subsequent open reading frames (orfs) (Fig. 1). The function of these proteins is unknown, but a BLAST against the GenBank database shows homology with pathogenicity island proteins. In addition, SaPI2 is present in the WKZ isolates, which encodes toxic shock syndrome toxin and exfoliative toxin A. The vSaα is identical for the WKZ isolates and MRSA252 with two exceptions. One exotoxin gene shows 3 nucleotide deletions leading to a slightly different C-terminal amino acid sequence for this protein (Supplementary figure 1). The second dissimilarity, which is in a protein part of a restriction modification system, contains 20 point-mutations which lead to 5 amino acid changes (Supplementary figure 2). In addition, the restriction modification system present in vSaß of MRSA252 is not functional in the WKZ isolates as the gene encoding the methylase is truncated.

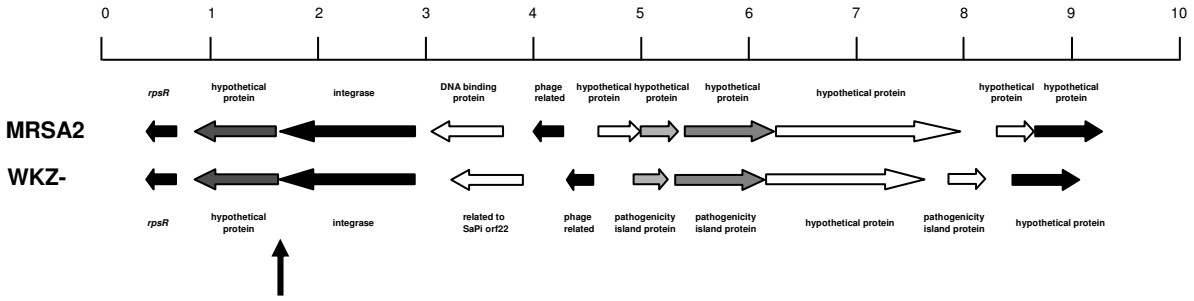

Fig 1. Alignment of the first part of SaPI2 from WKZ-1 (top) with MRSA252. Arrows indicate open reading frame possibly encoding proteins. White indicates no similarity between the proteins. The increasing greys scale indicates increasing similarity. Black arrows indicate identical sequences. Vertical arrow indicates the boundary between the *S. aureus* chromosome and SaPi2.

Finally, an additional gene for a SD-repeat protein (*sdrD*) is present in the WKZ isolates but not in MRSA252. Both MRSA252 and the WKZ isolates contain the *sdrC* and *bbp* gene, which are also SD-repeat proteins. The latter gene encodes bone sialoprotein binding protein.
No differences were observed in the sequences of WKZ-1 and WKZ-2, except for the presence of SCC*mec* in the latter isolate. The isolates can therefore be considered isogenic. The SCC*mec* of the proposed donor *S. epidermidis* isolate O7.1 belonged to type IVa. However, the SCC*mec* of WKZ-2 contains an additional sequence that is homologous to the 6987 bp cassette chromosome(SCC)-like element of strain ATCC25923 [9]. This element was already present in WKZ-1. The SCC*mec* IVa sequence of *S. epidermidis* O7.1 is identical to that of the WKZ-2 isolate except for

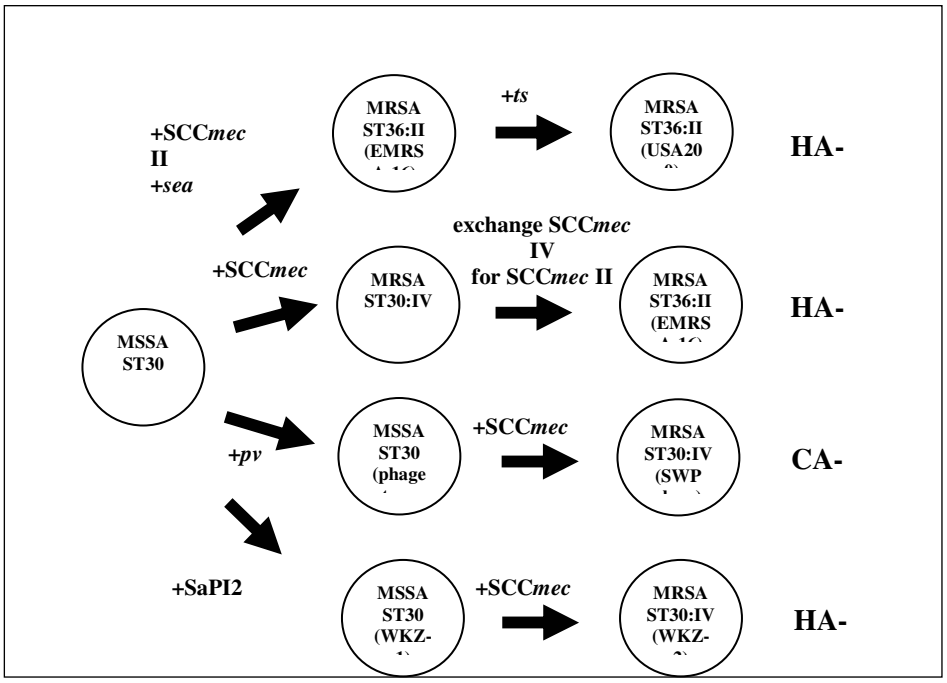

Figure 2. Proposed evolutionary routes for the SWP clone, and related HA-MRSA. For the emergence of EMRSA-16 Robinson et al. and Diep *et al*. propose different routes. The first group proposes a route in which SCC*mec* IV is acquired first, which is subsequently exchanged for SCC*mec* II., whereas Diep *et al*. propose direct acquisition of SCC*mec* II [18, 19]. In this study the ST30 MSSA had already acquired SaPI2 with the toxic shock syndrome toxin gene and subsequently acquired SCC*mec*.

a single point-mutation that resulted in a valine to alanine amino acid substitution in *ccrB*, which encodes a recombinase involved in the excision and integration of SCC*mec*. The lack of differences between WKZ-1 and WKZ-2 and the presence of only one point-mutation between the SCC*mec* elements of the proposed donor *S. epidermidis* and WKZ-2 strongly support our hypothesis that WKZ-2 was derived from WKZ-1 by transfer of SCC*mec* from *S. epidermidis* O7.1.

Table 1. Antibiograms

Antibiotic	MSSA WKZ-1*	MRSA WKZ-2	MRSE 07.1
Gentamicin	S	S	S
Cefoxitin	S	*R*	*R*
Ampicillin	S	*R*	*R*
Penicillin	*R*	*R*	*R*
Oxacillin	S	*R*	*R*
Amoxicillin-clavulanate	S	*R*	*R*
Trimethoprim-sulfamethoxazole	S	S	*R*
Trimethoprim	S	S	*R*
Teicoplanin	S	S	S
Vancomycin	S	S	S
Clindamycin	S	S	S
Erythromycin	S	S	S
Quinupristin-dalfopristin	S	S	S
Linezolid	S	S	S
Mupirocin	S	S	S
Nitrofurantoin	S	S	S
Ciprofloxacin	S	S	S
Levofloxacin	S	S	S
Moxifloxacin	S	S	S
Rifampicin	S	S	S
Tetracyclin	S	S	S

*S = susceptible

R = resistant

Resistance to antibiotics was studied. *S. epidermidis* 07.1 was resistant to cefoxitin, ampicillin, penicillin, amoxicillin/clavulanate and trimethroprim/sulfametoxazol. MSSA WKZ-1 was not resistant to any of the tested antibiotics. WKZ-2 MRSA was resistant only to oxacillin. Replication of the transfer from *S. epidermidis* to WKZ-1 using *in vitro* filter matings in the presence or absence of ß-lactam antibiotics was not successful.

Discussion

We previously reported the isolation of a closely related MSSA and MRSA and potential SCC*mec* donor *S. epidermidis* isolate from the same patient. Based on fingerprint techniques we hypothesized that the *S. epidermidis* had transferred SCC*mec* to the WKZ-1 MSSA to become the WKZ-2 MRSA [8]. In the present study, we performed whole genome sequencing on both isolates. The results show no difference in nucleotide sequence between the two WKZ isolates except for the presence of SCC*mec* in WKZ-2. A previous study had shown that SCC*mec* of WKZ-2 was bordered by the SCC-like element of *S. aureus* strain ATCC25923, whereas this element was lacking in the *S. epidermidis* strain. Sequencing of the SCC*mec* of the *S. epidermidis* showed that it was identical to the SCC*mec* of WKZ-2 except for a single point-mutation. The SCC-like element that is also present in WKZ-2 was also previously shown to be present in WKZ-1 [10]. In the current study we show that the sequences of the SCC-like element are identical in both isolates, except for a single point-mutation in the *ccrB* gene which leads to a valine to alanine substitution in the encoded recombinase.

Apparently, the WKZ strain is able to accept SCC elements rather easily. This strain has the ability to excise these SCC elements either independently or together, but excision at an alternative site was also noted [10]. A possible cause for the ease of the acceptance of SCC elements is the inactivation of a restriction modification enzyme in vSaß by the introduction of a frame-shift. In addition, the additional mutations present in the restriction modification system of vSaα may also influence the functionality of this system. Restriction modification systems are a way for bacteria to control the uptake of foreign DNA. The hypothesis that restriction modification systems influence the ability of different *S. aureus* strains to accept SCC element is also supported by data from Waldron and Lindsay [11], who showed that variation in the restriction modification system corresponded with the 10 major *S. aureus* lineages.

Based on the data of this study and data of Nübel *et al.*, which indicate that transfer of SCC*mec* into *S. aureus* is not as rare as previously believed [12,13], we tried to replicate the transfer of SCC*mec* from the *S. epidermidis* O7.1 to WKZ-1 in filter matings. However, these experiments were unsuccessful. This was also the case when the antibiotics used to treat the child were used to induce

transfer. This suggests that transfer of SCC*mec* to *S. aureus* requires special circumstances that we still do not understand in contrast to the situation in *Enterobacteriaceae* where transfer of resistance elements can be easily achieved both *in vitro* and in patients [14].

The WKZ-2 is likely to be more pathogenic than MRSA252, which is a representative of EMRSA-16, as WKZ-2 contains a number of additional virulence factors. Most notably, the WKZ-2 strain contains SaPI2, which encodes both toxic shock syndrome toxin and exfoliative toxin. Especially toxic shock syndrome toxin has been associated with severe disease [15], whereas exfoliative toxin A, which acts as a specific protease has been associated with skin damage [16]. The acquisition of SaPI may also be the result of the impaired restriction modifications systems. So, impaired restriction modification not only leads to the emergence of novel antibiotic-resistant strains, but also more pathogenic strains. In addition, the *sdrD* gene is present in WKZ-2 which encodes a putative adhesion protein. This may play a role in tissue specificity of the strain. A difference in a vSaq encoded exotoxin was also observed.

The WKZ isolates belong to the same clonal lineage as the community-acquired MRSA clone known as the Southwest Pacific clone [17]. The MSSA that stood at the basis of the SWP clone is also hypothesized to be the ancestor for EMRSA-16 to which MRSA252 belongs. The ancestor MSSA was proposed to first acquire a SCC*mec* IV, which was subsequently replaced by a SCC*mec* II [18]. However, Diep at al suggested that EMRSA-16 arose directly from MSSA ST30 by the acquisition of SCC*mec* II. From this strain USA200, the second most common hospital acquired MRSA in the USA, arose by acquisition of the toxic shock toxin gene [19]. In this study the ST30 MSSA had already acquired SaPI2 encoding the toxic shock syndrome toxin and subsequently acquired SCC*mec* (Fig. 2). The variety of different strains that arose from ST30 by the independent acquisition of different mobile genetic elements suggests that this lineage has a genetic background that is well-suited for the uptake of these elements.

In conclusion, the data presented strongly support our hypothesis that interspecies transfer of a SCC*mec* took place, in a patient undergoing antibiotic therapy, from *S. epidermidis* O7.1 to MSSA WKZ-1, to become MRSA WKZ-2. Our hypothesis is that MSSA WKZ-1 is highly receptive for mobile genetic elements, due to an alteration or impairment of two restriction modification systems. Unfortunately,

we were unable to replicate SCC*mec* transfer *in vitro*. This may indicate that the interspecies transfer of SCC*mec* is either relatively rare, or that the *in vitro* circumstances were unfavorable. Nevertheless, the presence of *S. aureus* strains with impaired restriction modification systems may lead to the emergence of novel and highly pathogenic strains.

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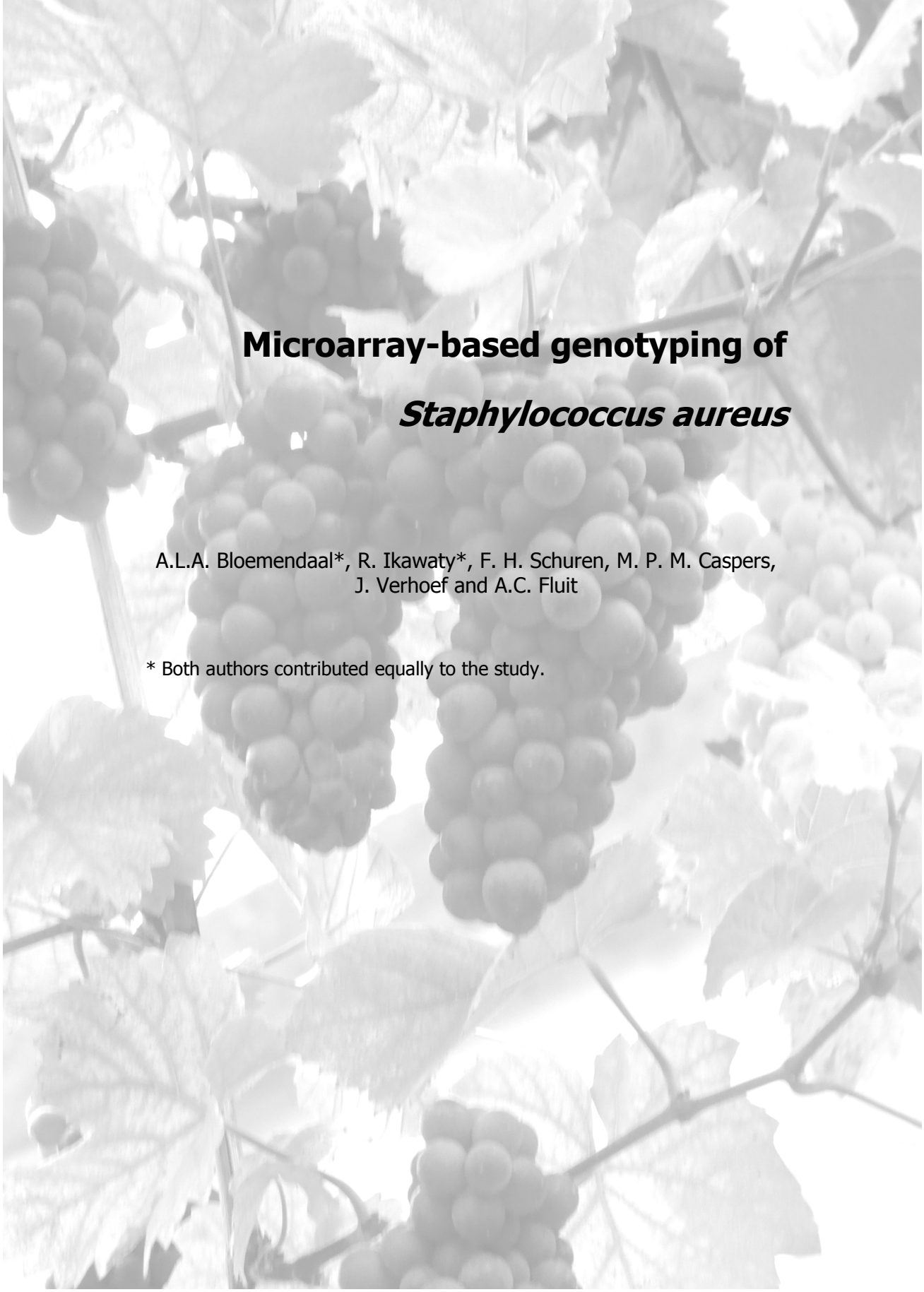
Supplementary figure 1. The alignment of the exotoxin of vSaß that differs between WKZ-1 and MRSA252.

```
WKZ-1
MKMTQIAKTSLALSLLTTGASIVTTQSANAEVASALKTEQAIKSKIQKVT   (  50)
MRSA252.TXT  ..................................................  (  50)

WKZ-1
TSPSTKVKATQPTPSVTTTTPPSPNVQPQSPQPTPNPTTPHSSNVETKQP  ( 100)
MRSA252.TXT  ....PN.QPQS.Q.TPSV.....................  ( 100)

WKZ-1
QSPTTKQAQKEINPKYKDLRTYYTKPSLEFEKQFGFMLKPWTTVRFMNVI  ( 150)
MRSA252.TXT  ..................................................  ( 150)

WKZ-1
PDWFIYKIALVGKDDKKYKDGPYDNIDVFIVLEDNKYQLKKYSVGGITKT  ( 200)
MRSA252.TXT  ..................................................  ( 200)

WKZ-1
NSKKVDHKAELSITKKDEKGKISHDDSEYKITKEEISLKELDFKLRKQLI  ( 250)
MRSA252.TXT  ..................................................  ( 250)

WKZ-1
EQHNLYGNIGSGTIVIKTKNGGKYTFELHKKLQEHRMADVIDGTSIERIE  ( 300)
MRSA252.TXT  ..................................................  ( 300)

WKZ-1        VNLKSS  ( 306)
MRSA252.TXT  ......  ( 306)
```

Supplementary figure 2. Alignment of the vSaα restriction modification protein of WKZ-1 and MRSA252.

```
WKZ-1
MSITEKQRQQQAELHKKLWSIANDLRGNMDASEFRNYILGLIFYCFLSEK  ( 50)
MRSA252      .....................................R.....  ( 50)

WKZ-1
AEQEYADALSGEDITYQEAWADEEYREDLKVELIDQVGYFIEPQDLFSAM  ( 100)
MRSA252      .........A.....................A.....  ( 100)

WKZ-1
IHEIETQDFDIEHLATAIRKVETSTLGEESENDFIGLFSDMDLSSTRLGN  ( 150)
MRSA252      .R.....  ( 150)

WKZ-1
NVKERTALISKVMVNLDDLPFVHSDMEIDMLGDAYEFLIGRFAATAGKKA  ( 200)
MRSA252      .....  ( 200)

WKZ-1
GEFYTPQQVSKILAKIVTDGKDKLRHVYDPTCGSGSLLLRVGKEAKVYRY  ( 250)
MRSA252      .....  ( 250)

WKZ-1
FGQERNNTTYNLARMNMLLHDVRYENFDIRNDDTLENPAFLGHTFDAGIA  ( 300)
MRSA252      .....V..  ( 300)

WKZ-1
NPPYSAKWTADSKFENDERFSGYGKLAPKSKADFAFIQHMVHYLDDEGTM  ( 350)
MRSA252      .....  ( 350)

WKZ-1
AVVLPHGVLFRGAAEGIIRRYLIEEKNYLEAVIGLPANIFYGTSIPTCIL  ( 400)
MRSA252      .....  ( 400)

WKZ-1
VFKKCRQQDDNVLFIDASNDFEKGKNQNHLSDAQVERIIDTYKRKETIDK  ( 450)
MRSA252      .....  ( 450)

WKZ-1
YSYSATLQEIADNDYNLNIPRYVDTFEEEAPIDLDQVQQDLKNIDKEIAE  ( 500)
MRSA252      .....  ( 500)
```



Microarray-based genotyping of *Staphylococcus aureus*

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Abstract

The impact of infections by *Staphylococcus aureus* in both hospital and community settings has led to intensive investigation of this organism. A number of typing methods have been developed for *S. aureus* depend on the research questions have been asked, aiming at population study or outbreak detection. However, none of the existing methods is able to answer both objectives. DNA microarrays have become more popular recently, due to their ability to detect thousands of distinct DNA sequences simultaneously. We evaluated a DNA microarray with 400 selected probes with 164 randomly chosen clinical human *S. aureus* isolates including outbreaks and 14 isolates that were taken over a 7 year time period from 4 patients. These isolates have been extensively genotyped by MLST, *spa*-typing and MLVA. In addition, 157 genotyped *S. aureus* isolates from 6 European ICUs were analyzed. Typeability, reproducibility and stability of this method were determined. The discriminatory power and epidemiologic concordance of the microarray were compared with the other methods. The microarray data demonstrated good typeability and reproducibility with a discriminatory power equal to MLVA. The microarray data reasonably correlated with the *spa*-typing and MLST results. In addition, the microarray was able to identify the outbreak isolates in both groups of isolates. Reduction to 205 probes yielded results similar to those obtained with 400 probes.

The same microarray data (but using a different cut-off for cluster separation) were also used to evaluate its potential for population studies. MLST was used as reference method. The microarray reasonably correlated with the MLST. However, the definition of the genetic distance used as a cut-off for the assignment of isolates to clusters for population studies is currently suboptimal. Thus, the present data imply that a microarray with a limited number of probes can be useful for typing *S. aureus* including detection of outbreaks and population structure analysis using different cut-offs. Inclusion of specific virulence factors or resistance genes may further enhance the utility of the microarray.

Introduction

Staphylococcus aureus is a major pathogen and much effort has been put in the development of typing methods to speed up and improve the characterization at molecular level. A variety of typing methods are available and the choice largely depends on whether *S. aureus* is studied at the population level or at the (local) outbreak level and on the availability of the method to the investigators. For local outbreaks Pulsed-Field Gel Electrophoresis (PFGE) has been considered as the gold standard since it has high discriminatory power, but this method is technically demanding, time consuming, expensive, and inter-laboratory data exchange is hard to achieve [3,26]. Recently, DNA sequence technology became more widely available and affordable. Therefore, sequence-based typing methods have been developed. These techniques are more commonly used in research laboratories with easy access to sequencing facilities [10]. Enright *et. al*. introduced a method to characterize strains of *S. aureus* based on the allelic profiles of seven house-keeping genes known as a Multi-Locus Sequence Typing (MLST). The results of MLST are completely reproducible, in digital format, and full access to a database (www.mlst.net) offers easy data exchange for MLST users worldwide. The MLST database currently contains information for more than 1700 isolates from 50 different countries [7,8]. But this method is only useful for population studies.

A relatively new method is *spa*-typing which is based on sequence variations in a region of the Protein A encoding gene of *S. aureus* (*spa*) containing 24 nucleotides long repeat units. Full access to a database is also available (www.SpaServer.ridom.de). Currently, it covers more than 4500 isolates from 47 countries. However, it has only limited utility for typing outbreaks, because some *spa*-types are common in the *S. aureus* population.
Multiple Locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA), a method based on the unique length of regions containing repetitive DNA loci, is another popular method and has been widely used for typing of a number of pathogens including *S. aureus* [1,12,14,15,18,25]. We have shown that a novel MLVA scheme is comparable with PFGE and has significantly higher discriminatory

power than *spa*-typing and MLST [15]. This method is useful for detecting outbreaks.

Genotyping of *S. aureus* by means of the DNA microarray technology has been reported earlier. The use of a microarray allows the simultaneous detection of large numbers of genes [17,19-23]. It may offer the possibility to study *S. aureus* both at the outbreak and population levels using the same dataset. However, available microarrays include far more probes than required for typing, e.g., probes against all core genome genes. This makes these arrays too expensive and complex for more routine applications.

We developed an approach that initially used a DNA microarray with 400 selected probes. This microarray was tested on 164 extensively genotyped human *S. aureus* isolates and 157 genotyped *S. aureus* isolates from patients admitted to 6 European intensive care units. Based on the data obtained the number of probes was reduced and a minimum set of probes required for outbreak detection was defined. Definition of different cut-offs was used to demonstrate the utility of the microarray data at both the outbreak and population structure level.

Materials and Methods

Bacterial strains
A total of 321 human *S. aureus* isolates were used for this study. These isolates were composed of two groups: a group of 164 isolates and a group of 157 isolates. Hundred isolates from the group of 164 isolates were taken from collection of the European Network for Antibiotic Resistance and Epidemiology (ENARE) at the University Medical Centre Utrecht in The Netherlands and represented 35 MLST types (STs) which were well distributed within the whole population of *S. aureus*, including the 5 major MRSA-clonal complexes (CC5, CC8, CC22, CC30 and CC45) and singletons. These 100 isolates were clinical isolates of which 25 were susceptible to methicillin (MSSA) and 75 were resistant to methicillin (MRSA). They were collected between 1997 and 2004. Fifty clinical methicillin-resistant *S. aureus* strains were obtained from the Department of Infection Control and Infection Prevention of the University Medical Centre Utrecht (UMCU), The Netherlands. They consist of 22 isolates that were considered to belong to 6 outbreaks, while 28 isolates were considered epidemiologically unlinked. Fourteen isolates from the Department of Infection Control and Infection Prevention UMCU, The Netherlands collected from 4 patients at different time-points were included. These isolates were genotyped by Multilocus Sequence Typing (MLST), staphylococcus Protein A (*spa)*-typing and Multiple Locus Variable Number Tandem of Repeat (VNTR) Analysis (MLVA) as shown in a previous study [15].

The second group of 157 *S. aureus* isolates was collected as part of the SEPTIC study to assess clonal spread on intensive care units (ICUs) in 6 countries in Europe, including The Netherlands, France, Portugal, Spain, Italy and Greece. During a 3 month period *S. aureus* isolates were obtained from all patients and typed by *spa*-typing and MLVA. Of these isolates 157 were selected based on MLVA-type (MT), *spa*-type, *mecA*, and ICU (country) to obtain a representative group of isolates [5].

Preparation of genomic DNA
Bacteria were grown on blood agar (Trypticase soy agar II containing 5% sheep blood) overnight at 37^{o}C prior to DNA isolation.

Preparation of bacterial genomic DNA was performed using the NucleoSpin kit (Machinery-Nagel) according to instructions from the manufacturer with the exception that bacterial pellet was resuspended in buffer T1. In our method, T1 buffer was replaced by freshly-made lysis buffer that contained 20 mM TrisHCl, 2 mM EDTA, 1% Triton X-100, and supplemented with lysostaphin, achromopeptidase and RNase. Purified DNA was measured with a NanoDrop spectrophotometer for its DNA concentration and stored at -20^{o}C prior to use.

Tools for comparison of microarray, MLVA, spa-typing and MLST

The discriminatory power of the typing methods was calculated by using EpiCompare version 1.0 (Ridom GmbH, Wurzburg, Germany). This software was also used for the determination of Adjusted Rand index and Wallace's coefficients.

Microarray construction

Four hundred probes chosen for the microarray were created and prepared at TNO Quality of Life Department of Microbiology, Zeist, The Netherlands. A mixed-genome microarray was constructed using genomic DNA of 8 *S. aureus* isolates (Table 1) in an approach similar to that used for *Enterococcus faecium*, the *Enterobacter cloacae* complex, and Group A streptococci [4,16,24]. Briefly, genomic DNA of the 8 isolates is sheared and 1-1.5 kb fragments are cloned into *Escherichia coli*. The inserts were amplified and 2112 inserts were spotted in duplo on the microarrays. After initial hybridization with DNA from 155 *S. aureus* isolates, 400 specific probes were selected as basis for a genotyping microarray.

Genomic DNA labeling

0.5 μg of genomic DNA from the strains to be tested was labeled with Cy5-dUTP (final concentration 0.06 mM, Amersham Bioscience) using the BioPrime DNA labeling system (Invitrogen). An equal amount of mixed genomic DNA used as reference strains was Cy3-dUTP labeled (final concentration 0.06 mM, Amersham Bioscience).

Table 1. Seven *S. aureus* isolates were used as reference strains for microarray.

Isolate	MSSA/MRSA	SCC*mec*	MLST type (ST)	*Spa*-type	MLVA type (MT)	Country of origin
S0021	MRSA	II	713	t018	24	Austria
S0025	MRSA	I	247	t051	41	Belgium
S0027	MRSA	IV	8	t009	32	France
S0029	MRSA	IV	8	t008	32	France
S0038	MSSA	-	617	new*	221	Germany
S0045	MRSA	IV	247	t052	27	Italy
S0049	MSSA	-	239	t275	26	Poland

* New *spa*-type observed and submitted to the Ridom SpaServer database (www.SpaServer.ridom.de) for assignment *spa*-type.

Blocking and prehybridization of microarray slides

After spotting, microarray slides were quickly washed three times in prehybridisation solution to block the slide with BSA and prevent spotted PCR products of binding to the non-spotted slide area. Then, slides were soaked in preheated prehybridisation solution (1% BSA, 5x SCC and 0.1 % SDS) and incubated at 42°C for 45 minutes while rotating. The microarray slides were washed in milliQ water, dried with a nitrogen flow and prewarmed at 42 °C.

Hybridization

The hybridization was performed on all 321 isolates. In addition, 17 out of the group of 164 isolates were randomly chosen and included for duplicate and triplicate analysis. In addition, 12 isolates of the ICU isolates were randomly chosen for duplicate analysis. Cy5- and Cy3-labelled genomic DNA were combined in equal amounts and 4 µl of 25 µg/µl yeast tRNA (Invitrogen) was added. The mixture was dried using a Speedvac at high temperature and dissolved in 40 µl Easyhyb (Roche). Denaturation of the hybridization mixture (target mixture) was performed by incubation at 100°C for 2 min and followed by hybridization of the target mixture to the prehybridized, prewarmed

microarray slides. Hybridization was performed overnight at 42 °C in hybridization chambers (Corning Life Sciences B.V.). On the next day, the hybridized microarray slides were washed with a concentration gradient of sodium chloride and sodium citrate solution and dried with a nitrogen flow [4]. The hybridized microarray slides were then ready for scanning and analysis.

Image analysis and data processing

A ScanArray Express (Perkin Elmer) was used for microarray image scanning. Microarray images were visualized and analyzed by using ImaGene version 5.6 (Biodiscovery) software for spot detection and quantification. The presence or divergence of genes was defined by the ratio of signal intensities from Cy5 (test strain) and Cy3 (reference strain) for all spots after local background signals were subtracted. The overall intensities of Cy5 and Cy3 channels were corrected by normalization. TIGR's (The Institute for Genome Research) Multi Experiment Viewer (TMEV) version 4.1. program was used for hierarchical clustering (HCL), genetic distance matrix (GDM) calculation, and significance analysis for microarrays (SAM).

Cluster determination based on microarray profiles

Genetic distance (GD) of each duplicate and triplicate (replicates) was measured by using distance threshold adjustment facility on the HCL. Median value of the genetic distance of replicates was used as a cut-off for the determination of the clusters at outbreak level. The genetic distance was independently calculated for each group of isolates. In addition, a higher cut-off was set (GD 8.1) for population level clustering determination.

Tools for comparison of microarray, MLVA, spa-typing and MLST

EpiCompare version 1.0 (Ridom GmbH, Wurzburg, Germany) was used to determine the discriminatory power, Adjusted Rand index, and Wallace's coefficients.

Probes reduction

The GD cut-off was used to define APs which then were used for SAM analysis. This was performed for determination of significant genes in order to reduce the number of probes required. This reduction was done independently for both groups of isolates.

Results

Genetic clustering of the 164 isolates based on microarray profiles

DNA of all 164 isolates could be hybridized. Performance of core genome normalization showed that some isolates yielded low signals, but no isolates were excluded from analysis. The duplicates and triplicates that were included showed similar profiles (Fig. 1). The genetic distance (GD) of the replicates as measured with the clustering software ranged between 4.79 and 8.1, while 6.5 was the median. A slightly higher cut-off of 6.6 was used for further analysis in order to include all clusters with GD 6.5. Analysis based on a cut-off of 6.6 for the 164 isolates with 400 probes generated 80 specific array profiles (APs).

Comparison of the Discriminatory Index for the microarray, MLVA, spa-typing and MLST

Discriminatory power of the microarray using 400 probes for the 164 isolates, in MLVA, *spa*-typing and MLST was determined and compared by calculating the genetic diversity (*D*) with 95% Confidence Interval (CI) of the 164 isolates typed by the four methods (Table 2). Simpson's indices of diversity for the microarray and MLVA were 0.985 and 0.987, respectively, indicating that the discriminatory power of both methods was similar using a GD cut-off of 6.6. In addition, the 95% CI of microarray is similar to that for MLVA. At this cut-off the microarray was more discriminatory than both *spa*-typing and MLST.

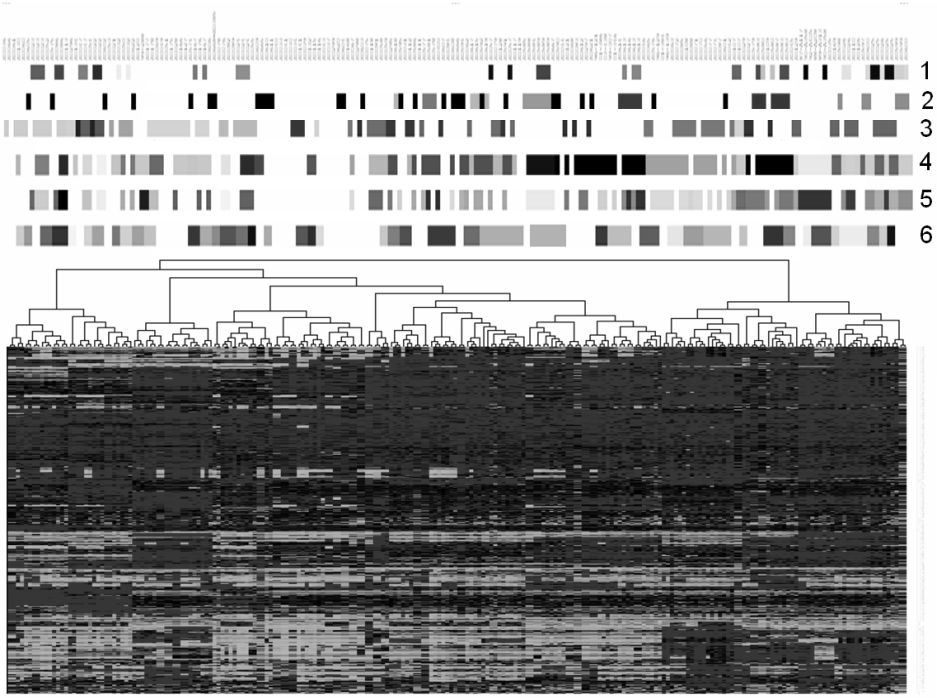

Fig 1. Two dimensional hierarchical clustering of the 164 *S. aureus* strains obtained from hybridization with 400 probes. Red and green spots represented the presence and divergence of genes (probes).The y-axis shows the 400 probes, while the isolates are on the x-axis. Clustering of the isolates was determined based on (1) replicates; (2) epidemiological relatedness; (3) MLST types; (4) *spa*-types; (5) MLVA types; and microarray profile (AP). APs were defined based on GD cut-off of 6.6. Different genotypes depicted by different colored bars on top of the dendrogram. (colorplate in Appendix II, fig 1)

The concordance between microarray, MLVA, spa-typing and MLST

The Adjusted Rand index for the microarray with 400 probes and the group of 164 isolates for microarray and MLVA, microarray and *spa*-typing, microarray and MLST was 0.253, 0.285 and 0.065, respectively (Table 3). The Wallace's coefficients given in Table 4 indicated that microarray was reasonably predictive for MLST and

spa-typing, but could poorly predict the MLVA, whereas the reverse was not true.

Table 2. Discriminatory power for 164 human *S. aureus* isolates of a microarray with 400 probes with a GD cut-off of 6.6, MLVA, *spa*-typing and MLST.

Typing method	Nr of different types	Discriminatory index	Confidence interval (95% CI)
Microarray	80	0,985	[0.981 - 0.99]
MLVA	104	0,987	[0.98 - 0.993]
***spa*-typing**	69	0,958	[0.944 - 0.971]
MLST	37	0,836	[0.785 - 0.887]

Table 3. Adjusted Rand index for 164 human *S. aureus* isolates of a microarray with 400 probes with a GD cut-off of 6.6, MLVA, *spa*-typing and MLST.

Typing method	Microarray	MLVA	*Spa*-typing	MLST
Microarray	-			
MLVA	0.253	-		
***Spa*-typing**	0.285	0.336	-	
MLST	0.065	0.041	0.151	-

Table 4. Wallace's index for 164 human *S. aureus* isolates of a microarray with 400 probes with a GD cut-off of 6.6, MLVA, *spa*-typing and MLST.

Typing method	Microarray	MLVA	*Spa*-typing	MLST
Microarray	-	0.253	0.588	0.552
MLVA	0.275	-	0.73	0.427
***Spa*-typing**	0.202	0.23	-	0.508
MLST	0.049	0.035	0.131	-

Comparison of the microarray data with outbreaks, MLST, spa-typing and MLVA

In general, there was agreement between the APs and the types defined by other typing methods (MLST, *spa*-typing and MLVA) for the group of 164 isolates including the outbreak groups. Almost all isolates assigned to an outbreak isolates also fell into the same AP.

An exception was found for an Outbreak 3 (O3) isolate (light green bar in Fig. 1, bar2) which did not cluster with the three other O3 isolates. It has AP 44, MT 63 and *spa*-type t2173 while others belong to AP 50, MT 27 and t002. Four Outbreak 6 isolates (depicted as a brownish-green bar in Fig. 1, bar2) had an identical MT 412 and t052. Three of them had an identical AP 76, one isolate, S0599 including its replicate, had an AP 79 which is slightly different from that of AP 76, and a replicate of isolate S0600 clustered in AP 80 instead of in AP 76 as expected. To be noted, the hybridization of isolate S0599, replicates of S0599 and S0600, were performed on different days.

In addition, 12 isolates obtained from 3 patients at different points in time showed clustering for each patient, with an exception of 2 isolates which were taken from patient 2 that had a different AP, as well as a different MT and ST (Table 5). These data demonstrate the long term stability of AP.

When the clonal complexes (CCs) were considered, there was no longer agreement between STs and APs. Depicted in Figure 1 bar 3, ST247 (dark-grey), ST239 (light-blue) and ST8 (black) belong to the CC8 , but they were distributed across the APs. This was also the case for ST228 (turquoise), ST225 (pink), ST15 (red), and ST5 (dark-blue) which belong to CC5.

Similar to the situation with MLST, isolates with identical *spa*-type did not always cluster together. As shown in Figure 1 bar 4, some isolates that belonged to t001 (blue), t002 (turquoise), t003 (light-pink) and t008 (black) were scattered across the dendogram.

Almost all isolates with an identical or closely related MT were found within the same main clusters, although a few exceptions were present. For example, Figure 1 bar 5 shows that some that isolates belong to MT 10 (light-pink), MT 32 (turquoise), and MT 27 (orange) were distributed across the dendogram.

Table 5. Genotyping of 14 *S. aureus* isolates collected from 4 patients over time using microarray, MLVA and *spa*-typing.

Patient	Isolate *	Microarray profile (AP)**	MLVA	*Spa*-type
1	96-121	59	63	8
	99-307	59	63	8
	01-386	59	63	8
	03-438	59	31	8
2	99-288	14	35	37
	01-346	60	56	121
3	87-A117	74	48	75
	88-A208	74	48	75
	89-A313	74	48	75
	90-A358	74	48	75
	92-A405	74	48	75
4	96-129	63	10	1
	96-139	63	10	1
	01-384	63	10	1

* : Numbering system of isolates indicating year of isolation and ID of the strains,
i.e. 96-121 means isolate collected in 1996 with isolate ID 121.
**: The AP was based on a GD cut-off of 6.6.

Genetic clustering of the ICU isolates based on microarray profiles using 400 probes

All 157 ICU isolates could be hybridized. The duplicates that were included showed similar APs (Fig. 2). The GD of the replicates ranged between 3.7 and 7.5, while 5.6 was the median GD. Analysis based on a cut-off of 5.6 for the 157 isolates with 400 probes generated 65 APs.

Comparison of the Discriminatory Index for the ICU isolates using the microarray, MLVA, and spa-typing

Discriminatory power of the microarray using 400 probes, MLVA and *spa*-typing was determined and compared as described above (Table 6). Simpson's indices of diversity for the microarray and MLVA were 0.982 and 0.988, respectively, indicating that the discriminatory power of both methods was similar. In addition, the 95% CI of microarray overlapped to that MLVA. Both microarray and MLVA had higher discriminatory power than *spa*-typing.

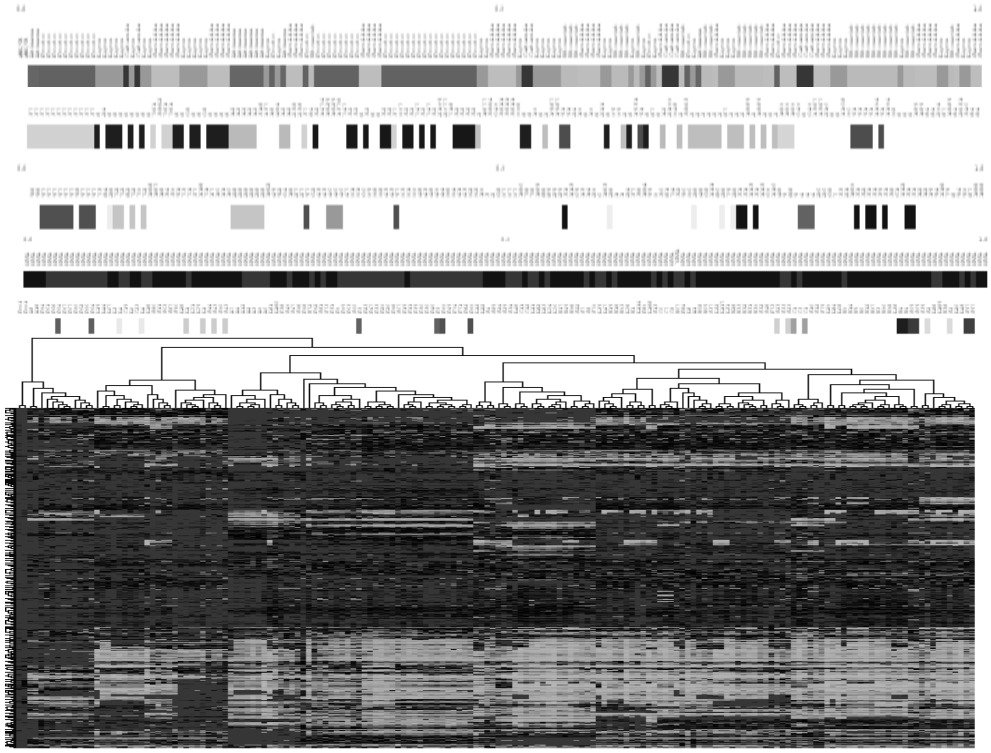

Fig 2.Two dimensional hierarchical clustering of 157 ICUs isolates using 400 probes. Shown from top to bottom clustering based on city of origin, *spa*-type, MLVA-type, MRSA/MSSA and duplicates. (colorplate in Appendix II, fig 2)

The concordance between microarray, MLVA, spa-typing for the ICU isolates

The Adjusted Rand index for the ICU isolates using the microarray with 400 probes for microarray and MLVA, microarray and *spa*-typing, 0.238, 0.241, respectively (Table 7). The Wallace's coefficients given in Table 8 indicated that microarray was poorly predictive for both *spa*-typing and MLVA. *Spa*-typing and MLVA were better predictors for the microarray (Table 8).

Table 6. Discriminatory power of the microarray with 400 and 205 probes with GD cut-off of 5.6, MLVA and *spa*-typing and MLST for 157 isolates from 6 ICUs.

Typing method	Nr of different types	Discriminatory index	Confidence interval (95% CI)
MLVA	102	0,988	[0.982 - 0.994]
***spa*-typing**	53	0,952	[0.937 - 0.967]
Microarray 400 probes	61	0,982	[0.977 - 0.986]
Microarray 205 probes	69	0,981	[0.974 - 0.988]

Table 7. Adjusted Rand index of MLVA and *spa*-typing compare to microarray using 400 and 205 probes with GD cut-off of 5.6 for 157 isolates from 6 ICUs.

Typing method	MLVA	*Spa*-typing	Microarray 400 probes	Microarray 205 probes
MLVA	-			
***Spa*-typing**	0,323	-		
Microarray 400 probes	0,238	0,241	-	
Microarray 205 probes	0,252	0,231	0,652	-

Table 8. Wallace's coefficient for 157 isolates from 6 ICUs genotyped by MLVA, *spa*-typing, and microarray using 400 and 205 probes with GD cut-off of 5.6.

Typing method	MLVA	*Spa*-typing	Microarray using 400 probes	Microarray using 205 probes
MLVA	-	0,767	0,206	0,218
***Spa*-typing**	0,217	-	0,185	0,435
Microarray using 400 probes	0,317	0,451	-	0,656
Microarray using 205 probes	0,333	0,179	0,661	-

Reduction of probes

From 400 biomarkers included for the analysis of the group of 164 isolates a set of probes was distilled by SAM that yielded the same number of clusters. It should be noted that this analysis used the set of APs defined by a GD cut-off of 6.6. A total of 103 probes (a 73% probe reduction) yielded an identical clustering as obtained with the original 400 probes. The same analysis for the 157 ICU isolates resulted in 157 differentiating biomarkers, a 60% probe reduction (Fig. 3 and 4). However, these two reduced genetic probe sets matched poorly. To obtain a set of probes, which was sufficiently discriminating in both experiments a comparison was made. A reduction to 205 probes (49% reduction) could be achieved.

Evaluation of a microarray using 205 selected probes

The 205 probes were tested on the 164 isolates. The GD cut-off was determined based on GDs replicates, ranged from 3.5 to 6.1, and a GD of 4.9 was the median. APs were determined based on a GD cut-off of 5.0 and resulted in 73 APs. Simpson's index of diversity of the microarray showed a comparable result to the microarray using 400 probes as shown in Table 2.

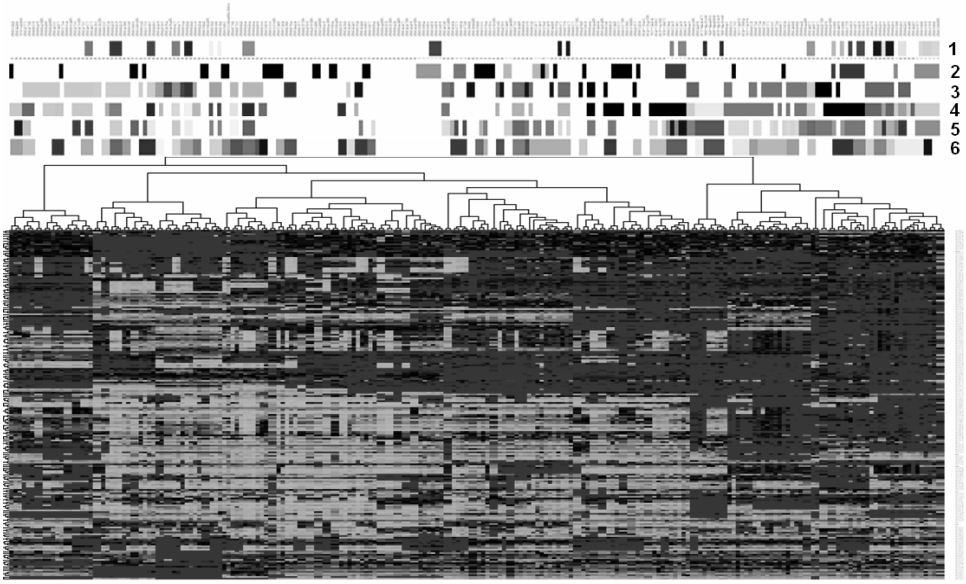

Figure 3. Two dimensional hierarchical clustering of 164 isolates with 205 probes after 45% of probes reduction (cut-off GD 6.6). Clustering of the isolates was determined based on (1) replicates; (2) epidemiological relatedness; (3) MLST types; (4) *spa*-types; (5) MLVA types; and microarray profile (AP) based on GD cut-off of 6.6. Color bars are identical to those of Figure 1. (colorplate in Appendix II, fig 3)

The 157 isolates were subjected to the microarray analysis using the minimum set of 205 significant probes. The duplicates showed identical APs. The GD cut-off was determined based on GDs of the replicates and ranged from 3.0 to 5.2 with a GD of 4.1 as the median value. Determination of APs based on a GD cut-off of 4.1 generated 69 APs.

The discriminatory index for the ICU isolates using 205 probes was comparable to the discriminatory index using 400 probes (see Table 2 and 6). The concordance between the reduced microarray and the 400 probe microarray for the ICU isolates was reasonable. The Adjusted Rand index was 0.652 (Table 7). The Wallace's coefficient

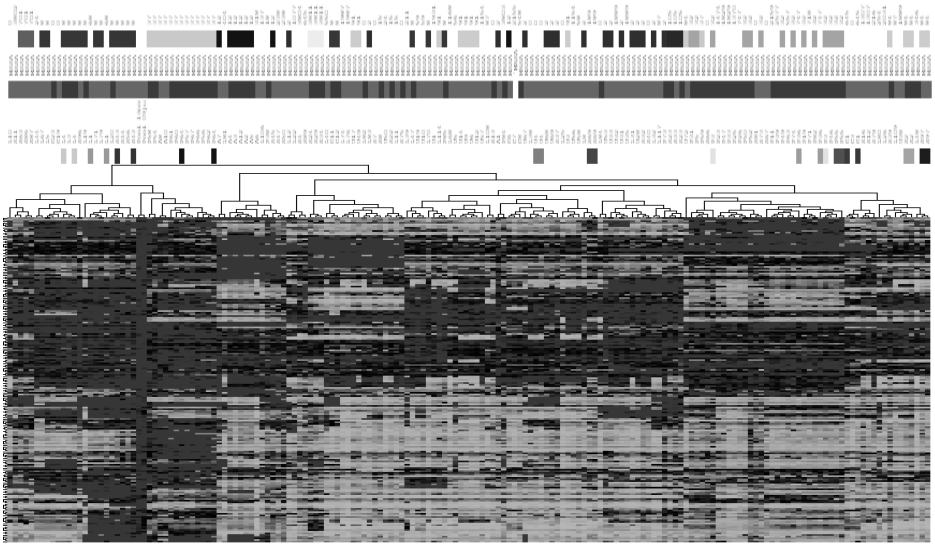

Figure 4. Two dimensional hierarchical clustering of 157 ICUs isolates using 205 probes. Shown from top to bottom clustering based on *spa*-type, MRSA/MSSA and duplicates. (colorplate in Appendix II, fig 4)

showed that the 400 probe microarray was a reasonable predictor for the reduced probe microarray, and vice versa (Table 8).

The Adjusted Rand index for the ICU isolates using the microarray with 205 probes and MLVA, microarray and *spa*-typing, was 0.25 and 0.231, respectively (Table 7). The Wallace's coefficients given in Table 8 indicated that microarray was poorly predictive for MLVA-typing and a slightly better predictor for *spa*-typing. *Spa*-typing and MLVA were poor predictors for the microarray (Table 8).

Clustering of APs for population study purposes

The maximum GD of replicates was 8.1 and a GD cut-off 8.2 was chosen for population study purpose. This resulted in 39 clusters. The Simpson's indices showed that the discriminatory power of this microarray was between MLVA and *spa*-typing with the 95% CI within the range of *spa*-typing (Table 9). The Adjusted Rand index and Wallace's coefficient indicated that the microarray could

reasonably predict MLST and *spa*-typing, but not for predicting the MLVA (Table 10 and 11).

Table 9. Discriminatory power of the microarray with 400 probes with a GD cut-off of 8.2, MLVA, *spa*-typing and MLST for the group of 164 human *S. aureus* isolates.

Typing method	Nr of different types	Discriminatory index	Confidence interval (95% CI)
Microarray	39	0.965	[0.958 - 0.973]
MLVA	104	0.986	[0.98 - 0.993]
***spa*-typing**	69	0.957	[0.943 - 0.971]
MLST	37	0.832	[0.779 - 0.884]

Table 10. Adjusted Rand index of four typing methods with a GD cut-off of 8.2 defined for the microarray with 400 probes.

Typing method	Microarray	MLVA	*Spa*-typing	MLST
Microarray	-			
MLVA	0.241	-		
***Spa*-typing**	0.375	0.341	-	
MLST	0.106	0.04	0.149	-

Table 11. Wallace's coefficient for 164 isolates genotyped by four methods. The data for the microarray with 400 probes are based on a GD cut-off of 8.2.

Typing method	Microarray	MLVA	*Spa*-typing	MLST
Microarray	-	0.179	0.447	0.46
MLVA	0.449	-	0.73	0.422
***Spa*-typing**	0.36	0.235	-	0.51
MLST	0.095	0.035	0.13	-

Discussion

Typing of *S. aureus* and particularly MRSA is far from ideal. Different methods suffer from different drawbacks and no method is able to yield data that can be used both for typing at the outbreak level and population structure level. Microarray technology may offer this possibility. Microarray technology has been intensively used because it allows the characterization of bacteria by determining the presence or divergence of thousands of genes in a single hybridization experiment. Typing would be based on the variability that (by definition) is provided by the accessory gene component of the bacterial genome.

The 164 clinical human *S. aureus* isolates used in this study including the outbreaks and the 14 isolates from 4 patients represented both the major clonal complexes as well as singletons from the whole *S. aureus* population. They have been extensively genotyped by MLST, *spa*-typing, and MLVA. In this study, these 164 isolates were hybridized with 400 probes. All isolates were successfully hybridized, although some of them obtained poor hybridization signals as shown by grey spots on the dendrogram (see Fig. 2). Similar APs were generally obtained for duplicates and triplicates, which indicates a good reproducibility of the microarray data. However, replicates of 2 isolates (S0599 and S0600) produced slightly different genetic profiles. These hybridizations were performed on different day and indicate slight variation in the results from day to day. Although normalization was performed to reduce the bias, the differences may be caused by slightly different experimental conditions such as batch or slide variations [9].

The use of genetic distance of replicates for defining the cut-off was a suitable method since it shows the actual genetic distance between isolates based on the microarray data. The genetic distance was defined as the dissimilarity of genetic profiles between isolates or individuals of the same species. The hybridization data of the 164 clinical *S. aureus* isolates were examined at two different levels: firstly for identification at the outbreak level, and secondly at the population level.

All isolates were typeable. Hybridization using a GD cut-off of 6.6 for the group of 164 *S.aureus* generated 80 APs. For the 157 ICU isolates a cut-off at GD of 5.6 was defined, which resulted in 65 APs. Simpson's indices of diversity for the microarray, MLVA, *spa*-typing and MLST showed that the number of types obtained with the microarray was comparable with that for MLVA and higher than for *spa*-typing and MLST. Clustering at the outbreak level was comparable with that obtained for other methods as demonstrated by the collection of 50 isolates that were either considered to belong to outbreaks or to be epidemiologically unrelated isolates. Only a few isolates were not assigned in accordance with their assignment by the Department of Infection Control and Infection Prevention. This can be caused by a number of reasons. In a previous study we also showed variation in MT within an identical *spa*-type or ST. We also observed variations in MT in isolates considered to belong to the same outbreak [15]. This suggests that variation between closely related isolates exists. This is likely due to (minor) variation in the accessory genes. The accessory genes are "unstable" since they are more prone to gain and loss of genetic information between closely related strains [2,6]. Another explanation is a low signal for some isolates, e.g., S0600. In addition, some probes did not yield a clear present/divergent result. This may be the result of cross-hybridization with for example probes that represent enterotoxin genes. Exclusion of these probes in an improved version of the microarray will also enhance the performance of the microarray. Also the reproducibility of the method needs to be improved as indicated by the variation in the some of the replicates, although reproducibility improved during the study as indicated by the lower GDs for the set ICU isolates for which hybridizations were performed later in the study. For this reason outlying results from triplicates were eliminated for the determination of the GDs. However, the assignment of the 14 isolates that were collected over a 7 year time period matched earlier assignment based on other typing methods [15]. Also the assignment of the isolates from the ICUs were grouped in accordance with previous typing results [5].

Significant genes in this set of microarray experiments were determined by a statistical technique SAM. Probe reduction for each group of isolates yielded sets of probes that matched only partly. This apparent discrepancy is caused by the different backgrounds from

which the isolates were collected. A set of approximately 200 probes yielded a clustering for each group of isolates that was comparable with that obtained by using 400 probes. This indicates that 400 probes are more than sufficient to type these isolates. However, it should be noted that with the addition of other isolates the approximately 200 probes may prove insufficient, despite the fact that we tried to include a diverse collection of strains based on MLST.

MLST has been widely used to study the population of *S. aureus* [8,11,13], and it clearly discriminates distinct lineages [17]. Here we attempted to use the dataset obtained with 400 probes and the 164 isolates for studying the population structure of *S. aureus*. A higher GD cut-off of 8.2 was applied and it resulted in 39 APs. Since the microarray could reasonably predict MLST, we conclude that the microarray can be used for population studies, but the determination of a correct cut-off should be improved.

It can be concluded that the use of a microarray with a limited number of probes is a useful addition for typing of *S. aureus* including MRSA. Nevertheless, the quality of the array should be improved by removing unstable probes, i.e., probes that can not be called present or divergent in an unambiguous way. Probes for specific virulence factors or resistance genes may further enhance the utility of the microarray. However, the major advantage of the microarray is that the data which are generated can be used for both outbreak identification and population structure analysis using different cut-offs.

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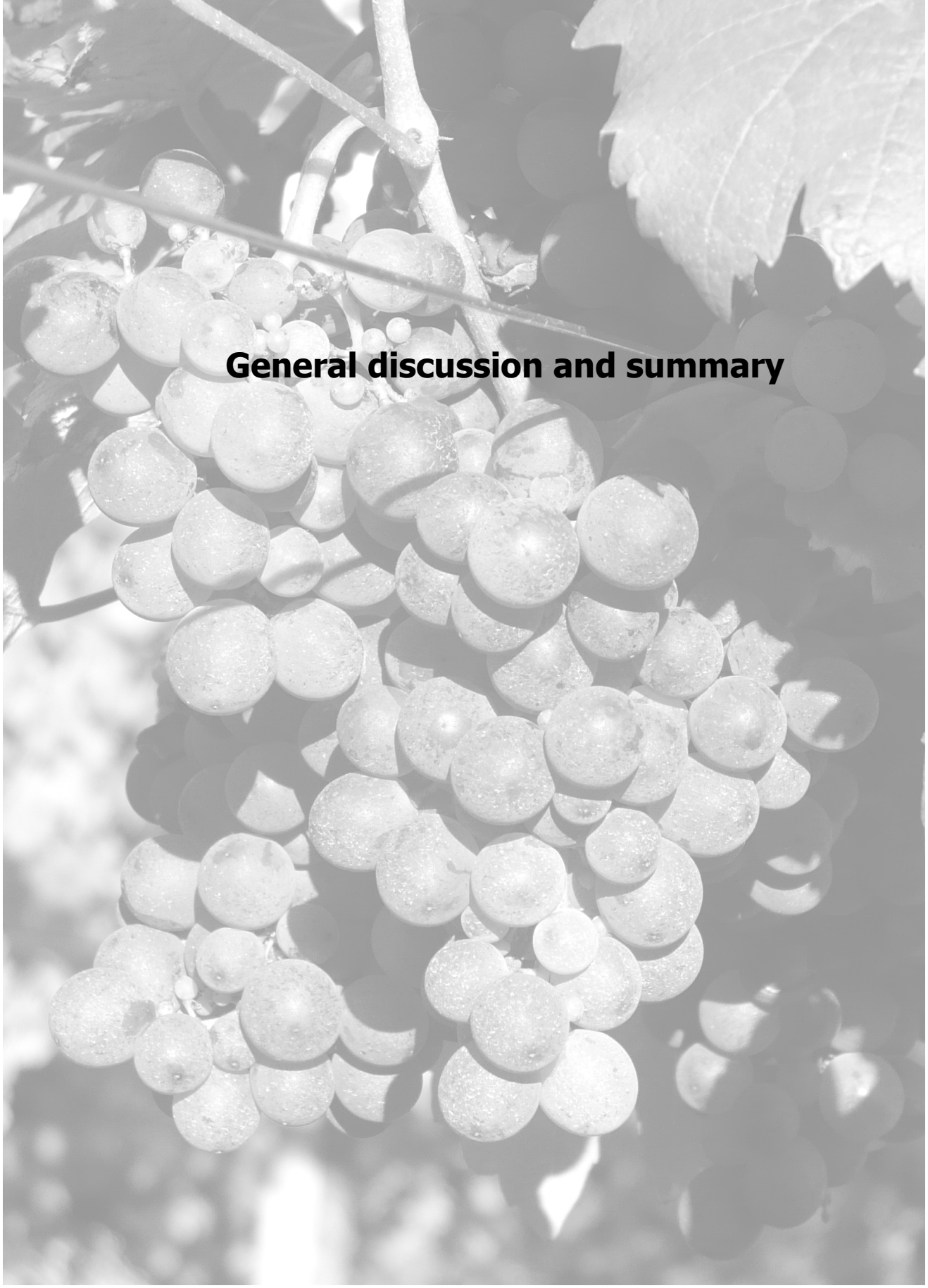

General discussion and summary

Discussion

Patients admitted to a hospital are at a considerable risk of acquiring a nosocomial infection. Overall prevalence of nosocomial infections is between 3.5% and 12% [1-8] and 30% to 40% of all patients admitted to an ICU will have or acquire an infection [5,9-11]. These infections lead to higher patient mortality and a considerable increase in healthcare costs. [12-15] Up to 30% of these ICU related infections are caused by *S. aureus* [8,9,16].
Nasal colonization by *S. aureus* is one of the most important factors in the pathogenesis of nosocomial infections, mainly in surgical site and vascular device related infections [17-19]. *S. aureus* generally colonizes human skin and mucous membranes. The anterior nares are the most frequent site of carriage [20]. Approximately 30% of the general population will be colonized by *S. aureus* at a certain point in time [21] and three types of carriership can be distinguished: persistent carriers (±20%), intermittent carriers (±30%) non-carriers (±50%) [21-24]. Nasal colonization rate by *S. aureus* among hospitalized patients is comparable to that in the general population. However, MRSA carriage among the general population is less than 1%, [25] whereas MRSA prevalence in nosocomial bacteraemia is over 50% in certain countries [26].

Antibiotic pressure [27-30], length of stay on an ICU [31-33], colonization pressure [33-35], healthcare-professional workload [35], hand hygiene level [35-38], isolation treatment measures [34,39-41] and environmental contamination [42-45] have been proposed as important factors in the acquisition and transmission of *S. aureus* on ICUs. Unfortunately, the importance of each individual factor remains unclear, as results have been highly variable. Moreover, the abovementioned factors are not uniformly defined and studied in different studies. Next to this, the importance of direct spread of distinct *S. aureus* strains between patients admitted to an ICU remains

undecided. A number of studies has shown low patient-to-patient cross-transmission of MRSA on ICUs [46-48], whereas other studies show a high relevance of cross-transmission [49-51].
These results were mainly obtained in single-center studies that assessed MRSA only. Unfortunately, it is unclear whether the considerable variations in rates of acquisition and cross-transmission are attributable to differences in *S. aureus* prevalence and infection-control measures or merely reflect variations in study-protocol and definition of influencing factors.

In an attempt to rule out the effect of abovementioned variations in study protocols we have performed a study on acquisition and cross-transmission of *S. aureus* (MSSA and MRSA) on different ICUs using one study-protocol. In chapter 2 we describe our findings.
Many studies have addressed the use of antibiotics and the number of nosocomial MRSA infections and ICU MRSA acquisition. The current consensus is that the amount of antibiotics prescribed to ICU patients is strongly correlated to the number of MRSA nosocomial infections and the number of MRSA acquisitions, especially for fluoroquinolones and macolides [27,28,52-57]. In one study the correlation between antibiotic use and the number of ICU infections was stronger in MRSA than MSSA [27]. We could not show a correlation between the amount of antibiotics used on an ICU and the acquisition or transmission of *S. aureus*. No difference was seen between MSSA and MRSA. This result is probably due a too small number of patients in our analysis. Two of the participating centers did not obtain information on antibiotic use, leading to a diminished analytical power. Our result concerning the correlation between antibiotic pressure and *S. aureus* acquisition is questionable.
A longer length of stay (LOS) on an ICU has been proposed as an important epidemiological factor, increasing the number of nosocomial MRSA infections and acquisitions [32,58,59]. However, these results were not confirmed by Grundmann et al. [31]. In our

results, length of stay was not shown to correlate with an increased chance of acquiring an *S. aureus*. Interestingly, patients acquiring an *S. aureus* (both MSSA and MRSA) remained on the ICU significantly longer than patients who did not acquire an *S. aureus* during stay, or who were admitted already colonized by *S. aureus*. This is probably due to the fact that patients with poor health status are more prone to acquire colonization by *S. aureus* [31-33,59].

In our study colonization pressure correlated positively to acquisition of both MSSA and MRSA, which is in concurrence to previously shown [33,60]. This correlation was not found by other studies [34,35]. Unfortunately, there is no consensus in the definition and calculation of colonization pressure. We assessed the mean number of patients colonized during a three day period prior to an acquisition to calculate colonization pressure. Merrer et al. and Dancer et al. calculated the mean number of colonized patients during each week and then assessed the number of acquisitions during that week. This could lead to a falsely high colonization pressure considered for an acquisition; a colonized patient may be admitted to the ward on a later day in the same week, increasing colonization pressure for that week, while this colonization is irrelevant for the actual acquisition.

Hand disinfection is an important hygiene control measure in the reduction of bacterial spread in hospitals. A number of studies has shown a negative correlation between hand hygiene compliance and acquisition of MRSA in ICUs [32,38,61,62]. In our results the average amount of hand disinfectant used correlated negatively to the number of acquisitions of MSSA. However, this was not found to be true for MRSA. The compliance to hand hygiene varied widely between the participating centers, ranging from an average 27 doses per bed per day to 97 doses. This wide variation cannot be explained and clearly weakens our results. The relevance of these results should be doubted. Hand hygiene remains mainstay in infection control.

A higher average number of beds per nurse (workload) was a significant risk factors for acquiring both MSSA and MRSA colonization. This result is supported by other studies [31,35,63,64]. A plausible explanation for this finding is a lower compliance to infection control measures during a relative shortage of personnel caring for the patients, i.e. decreased cohorting of nurses and a lower compliance tot hand hygiene protocols.
Treatment of ICU patients in isolation of other patients is an important infection control measure [40,41,65-68]. However, one large study showed no effect of isolating MRSA carriers on patient-to-patient MRSA cross-transmission [34], although patients were only placed in isolation after a positive culture for MRSA. During the days between the taking of the nasal swab and the finding of the positive MRSA culture the patient was already carrying the MRSA, possibly spreading the pathogen to other ICU patients. Cooper et al. reviewed current literature on isolation treatment of MRSA positive patients and concluded that no conclusive answer could be given on the effect of this measure, mainly due to methodological weakness of most studies in the assessment of isolation treatment as an individual infection control measure [39]. In our study, isolation treatment was correlated to a lower number of MRSA acquisitions, yet it was not correlated to MSSA acquisition. A definite reason for this difference in effect of isolation treatment on MSSA and MRSA acquisition cannot be given. An explanation could be that the number of MSSA carried by visiting family or consultants is higher than the carriage of MRSA by these contacts, leading to a higher MSSA pressure and a therefore a higher MSSA acquisition.

It has long been believed that nasal *S. aureus* carriage is clonal, especially in persistent carriers. This is mainly based on an old review [20], which based results on phage-typing. Vandenbergh et al. studied a large group of patients over a 10 year period and concluded the opposite; only a small minority of patients carries one distinct *S.*

aureus strain [24]. Other studies have shown polyclonal carriage within (non-) hospitalized patients and even simultaneous MRSA and MSSA carriage in one patient [69,70]. Lim et al. demonstrated intermittent, inconsistent and polyclonal MRSA carriage on different anatomical sites in ICU patients [71]. In chapter 3 we studied *S. aureus* carriage-clonality during ICU stay. Our results show that 8% of ICU patients carry 2 or more distinct *S. aureus* strains during their ICU stay. This result supports previous findings [70]. Furthermore, 1% of these patients carried both MSSA and MRSA, which were not clonally related. In one case, a patient carried six distinct *S. aureus* strains during ICU stay. We did not analyze multiple colonies from individual positive culture swabs. Therefore, simultaneous carriage of distinct strains could not be assessed. Next to this, our results show that only 45% of culture swabs taken from proven *S. aureus* carriers were positive for *S. aureus*. The question arises whether this is due to a loss and regain of nasal colonization over time, or due to a low analytical sensitivity of this method. Lim et al. concluded that a low consistency in colonization during ICU stay should be explained by technical failure in culture methods. We believe that the sensitivity is low due to inadequate swab taking. The fact that all swabs were cultured overnight in enrichment broth in our study should decrease the possibility of a technical failure due to laboratory methods. However, these results, supported by other authors, do call for an evaluation of swab taking and culturing methods as the gold standard in infection control. Although we cannot present an alternative at this time, a possible lack in analytical sensitivity of the present method should be taken in account.

Using DNA microarray it is possible to identify pathogens for hospital outbreak and infection control programs and for population structure analysis. In chapter 6 we describe the development of a 400 probe microarray for *S. aureus*. To this end 164 clinical human *S. aureus* strains, which were extensively typed in a previous study [72], were

analyzed by microarray to compare microarray profiles with MLST, MLVA and *spa*-typing. Furthermore, 157 clinical strains, obtained from 6 ICUs throughout Europe, as described in chapter 2, were subjected to this microarray for verification of the results. In this verification step the microarray results were compared to MLVA and *spa*-typing results. Microarray profiles (APs) were defined based on the combination of presence and divergence of probes. Microarray profile clustering was determined by genetic distance (GD). To obtain the GD replicates were used. Different GDs could be applied to perform outbreak analyses (high discriminatory power) or population structure analyses (low discriminatory power).

The Simpson's index of diversity showed a high typability and discriminatory power for the microarray. The discriminatory power was comparable to that of MLVA and higher than *spa*-typing and MLST. The epidemiological concordance analyses, performed using the Adjusted Rand Index and Wallace's Coefficient, showed a reasonable predictability of MLVA, *spa*-typing and MLST by microarray. Most importantly, the microarray classified outbreak strains correctly, proving its applicability in hospital infection control. Using a less discriminatory GD, we assessed the applicability of microarray for population studies. MLST was used as reference method as it is widely accepted to be the most valuable method in studying *S. aureus* lineages. The predictability of MLST by microarray was reasonable and we conclude that microarray is therefore applicable for population studies.

In an attempt to decrease the number of significant probes in order to obtain higher effectiveness of the microarray we applied a statistical technique termed Significance Analysis for Microarray (SAM). We found a minimum of 205 significant probes were required to obtain a similar result for epidemiological concordance and discriminatory power in comparison with the 400 probe microarray.

These results show the applicability of microarray in both hospital infection control and population structure studies, using one analysis

method and dataset. Enhancing clinical relevance of the microarray by adding probes for specific virulence and resistance elements could make microarray even more attractive in replacing the present "gold-standard" typing methods.

In chapter 4 we describe the whole genome sequences of an isogenic pair of MSSA and MRSA isolates (WKZ-1 and WKZ-2 resp.), which have been described previously [73]. In this study we show the transmission of a type IV SCC*mec* from a *Staphylococcus epidermidis* to a *S. aureus* in a newborn child, who developed a pneumonia days after birth. The initially isolated pathogen was a MSSA, for which the child was treated successfully with antibiotics. On day 32 after onset of the pneumonia a MRSA was isolated, which contained a SCC*mec* found in an *S. epidermidis* carried by the same patient.
The WKZ-1 and -2 isolates are fully isogenetic, except for the SCC*mec*. The closest comparable MRSA is MRSA-252 which had been genetically deciphered previously [74]. A number of differences was found. The WKZ-2 MRSA is ST30 and MRSA 252 is ST36. Both belong to CC30. The WKZ strain contains SCC*mec* type IV and MRSA 252 SCC*mec* type II. On WKZ-2 SAPI-2 is present, which contains the *tst*-1, encoding for toxic shock syndrome toxin. MRSA 252 contains a number of additional insertion sequences and resistance genes (e.g. Tn*554*).

One may presume that the epidemicity (transmission, colonization and colonization persistence) of MSSA should not differ from that of MRSA, as both strains are essentially identical, apart from the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*). A number of studies has been performed on fitness cost of resistance acquisition by different pathogens. Ender et al. showed a reduced growth rate after integration of a type I SCC*mec* in an MSSA in a direct competition growth experiment [75]. Lee et al. showed a fitness cost after type I SCC*mec* incorporation, but not after type IV SCC*mec.*

This was assessed by a glucose uptake and a growth rate experiment [76]. Karauzum et al. showed a decreased fitness in a ST8 MRSA compared to a ST8 MSSA, but not in a ST5 MRSA and MSSA (both type IV SCC*mec*) in an adhesion model and a murine sepsis model [77]. Noto et al. showed an increased fitness after SCC*mec* deletion in a VISA [78]. A decrease in fitness was also seen after acquisition of resistance to other antimicrobials [79-82]. In theory, the SCC*mec* cassette may encode for an increased or decreased epidemicity. This effect may also be achieved through polar effects due to the insertion of SCCmec into *orfX* on the staphylococcal chromosome. Genes on the SCC*mec* may influence regulatory peptides through an unknown mechanism.

In chapter 5 we describe the development of a murine epidemicity model to study the transmission and colonization persistence of *Staphylococcus aureus*. To this end we adapted a murine nasal colonization model described by Kiser et al. [83]. We used the abovementioned MSSA and a MRSA strain (WKZ-1 and WKZ-2 respectively) MSSA was shown to transmit more rapidly between mice than MRSA. However, there was no significant difference in the number of colonized mice on day 40. The number of colony forming units (CFUs) was found to be significantly higher in MRSA than MSSA colonized mice, suggesting a more stable colonization by MRSA, compared to MSSA. These results suggest a role for SCC*mec* in the transmission and colonization persistence of *S. aureus*. A definitive explanation cannot be given. As mentioned above different theories could be proposed to explain a difference in epidemicity between MRSA and MSSA, of which none have been studied yet.

Summary

Colonization pressure and number of beds per nurse are risk factors for the acquisition of both MRSA and MSSA on ICUs. Single room treatment of ICU patients decreases the chance of acquiring an MRSA. Eight percent of ICU patients carry two or more distinct *S. aureus* strains during ICU stay. Whether this is due to simultaneous polyclonal carriage or to colonization by different strains overtime is unclear. The fact that only 45% of culture swabs taken from *S. aureus* carriers is positive for *S. aureus* raises the question whether the nasal swab lacks analytical sensitivity. We developed a murine nasal transmission model for *S. aureus*. The number of mice to which MSSA had spread on day 5 was significantly higher than MRSA. However, at day 40 there was no difference in the number of colonized mice. The number of CFUs was significantly higher in MRSA colonized mice on day 40. These results suggest a more rapid spread by MSSA and a more stable colonization by MRSA, which can only be explained by an influence of SCC*mec* on the epidemicity of *S. aureus*. In this animal model we used the isogenic WKZ-1 and WKZ-2 strains. WKZ-1 (MSSA) acquired a type IV SCC*mec* from a *S. epidermidis* carried in the same patient during extensive antibiotic treatment for pneumonia to become an MRSA (WKZ-2). Microarray is a useful tool for typing *S. aureus*. It can be used in outbreak situations and for population structure studies. Furthermore, the addition of virulence and resistance genes could lead to a clinically more relevant application of microarray, in addition to the infection control application.

Implementation of aggressive infection control measures is the mainstay in containing the global spread of MRSA, VISA/VRSA and other multi-resistant pathogens and preventing as many nosocomial infections as possible. The results shown in the SEPTIC study are not conclusive for the identification of factors influencing *S. aureus*

transmission and infection on ICUs. A far larger study, using a single study protocol should be undertaken to decide whether future ICUs should contain private rooms only. Hand hygiene compliance should be stimulated at all times. Health care worker workload should be limited to guarantee adequate cohorting of nurses. Further investigations on virulence and epidemicity of *S. aureus* must be performed. To this end the murine transmission model, described in this thesis, could provide a valuable tool in the assessment of the influence of different genetic knock-outs, -downs or –ins on S*. aureus* transmission. The implementation of microarray as a tool for epidemiologic and outbreak studies awaits further validation. Microarray is relatively expensive, but the price will decrease rapidly once this typing-tool is implemented more extensively.

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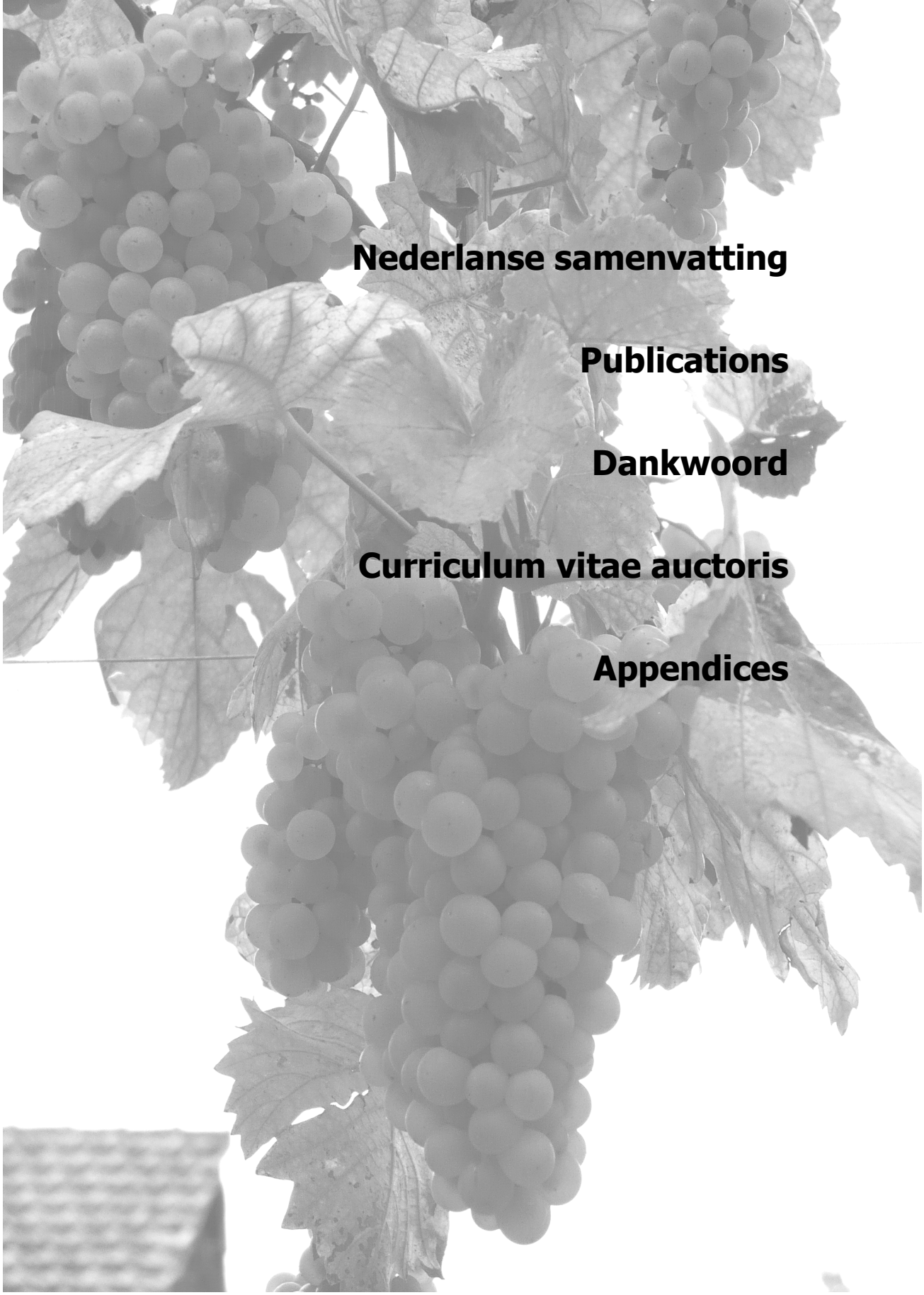

Nederlandse samenvatting

Staphylococcus aureus is één van de belangrijkste verwekkers van ziekenhuis gerelateerde en in het bijzonder intensive care unit (ICU) gerelateerde infecties. Deze nosocomiale infecties worden in 30% van de gevallen veroorzaakt door *S. aureus* en verhogen de ziekenhuis- mortaliteit significant. Daarnaast wordt de behandeling van *S. aureus* infecties bemoeilijkt door het ontstaan van resistentie tegen antimicrobiële middelen, waarbij methicilline resistente *S. aureus* (MRSA) de bekendste en meest onderzochte is.

In dit proefschrift beschrijven we het onderzoek naar verschillende factoren, die een rol spelen bij de verspreiding van *S. aureus* (inclusief MRSA).

In het eerste onderzoek richtten wij ons op de verspreiding tussen patiënten die opgenomen liggen op verschillende ICUs in Europa. In 6 ICUs in 6 landen werden bij alle patiënten herhaaldelijk kweken afgenomen. Gevonden *S. aureus* isolaten werden getypeerd middels moderne technieken, zodat een beeld verkregen werd van de verspreiding van specifieke stammen en het effect van verschillende hygiënemaatregelen, zoals hand-desinfectie, isolatiebehandeling en antibioticabeleid, hierop [*Infection Control & Hospital Epidemiology 2009 Feb;30(2):117-24*]. In dit onderzoek vonden wij ook, dat individuele patiënten verschillende *S. aureus* stammen bij zich kunnen dragen [*Infection Control & Hospital Epidemiology 2009 Sep;30(9):918-20*]. Dit zou consequenties kunnen hebben voor antibiotische therapie.

In het tweede onderzoek hebben wij een diermodel (in muizen) ontwikkeld voor de nasale kolonisatie en verspreiding van *S. aureus*. In dit onderzoek hebben wij ook het verschil in epidemiciteit van

methicilline gevoelige *S. aureus* (MSSA) versus methicilline resistente *S. aureus* (MRSA) onderzocht. Hiertoe hebben wij gebruik gemaakt van een genetisch isogene set MSSA en MRSA isolaten, waarvan wij in een apart onderzoek de genetische sequenties volledig hebben bepaald en geanalyseerd. Uit dit diermodel kwam naar voren dat MSSA zich sneller en gemakkelijker verspreid tussen de muizen, maar dat na verloop van tijd, de MRSA een stabielere en langdurigere kolonisatie teweegbrengt. Dit onderzoek is gepresenteerd tijdens het Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in Chicago in 2008.

Het laatste onderdeel van dit onderzoek bestond uit het ontwikkelen van een microarray om *S. aureus* genetisch te typeren. Met een microarray is het mogelijk de aan- of afwezigheid van een grote groep genen tegelijkertijd aan te tonen. Het grote aantal genen, dat men kan onderzoeken, geeft de mogelijkheid in één test zowel klinisch relevante genen (resistentie en virulentie genen) als epidemiologisch relevante genen te onderzoeken. Deze laatste toepassing kan gebruikt worden om op ziekenhuis, regionaal, nationaal en internationaal niveau de verspreiding van *S. aureus* en met name MRSA stammen te volgen. De eerste toepassing zou kunnen leiden tot sneller instellen van adequate antibiotische therapie.
Deze microarray is ontwikkeld in samenwerking met het TNO.

Publications

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toch weer overtroefd! Het worden twee mooie feesten. Peet, hoe vaak hebben wij aan de bar van een of andere Utrechtse kroeg geklaagd over ons onderzoek, onze promotoren, onze alles. En veel lol gehad! Het is fantastisch om een vriend als jij te hebben! Voor het leven.

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Pa en ma, ik hou van jullie!

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bewijs, dat échte tegenpolen, ondanks alle strijd die het oplevert, écht van elkaar kunnen houden. Wit heeft zwart nodig om wit te zijn en andersom. Jij bent mijn wit! Lief, ik hou van je! En sorry voor mijn agenda…

Curriculum Vitae Auctoris

Bob Bloemendaal was born in The Hague on 24th of March 1979 and grew up in Zevenhuizen, the Netherlands. He attended secondary school in Gouda (Coornhert Gymnasium) and graduated in 1998. The same year, he started Medical School at the University of Utrecht, the Netherlands. During his study his interest in Surgery grew and he completed 2 internships in this field (prof. dr. I.H.M. Borel Rinkes, UMC Utrecht and dr. V.J. Verwaal, Antonie van Leeuwenhoek hospital, Amsterdam). After graduating on 6th October 2005 he joined the Department of Surgery and the Department of Medical Microbiology to perform his PhD-studies (prof. dr. J. Verhoef, microbiology and prof. dr. I.H.M. Borel Rinkes, surgery), which has led to this thesis. On 1st July 2008 he has started as resident in General Surgery in the UMC Utrecht (prof. dr. I.H.M. Borel Rinkes). Currently he is working as a resident at the Meander Medical Center in Amersfoort (dr. A. van Overbeeke).

Appendix I

Table 5. Genotypes acquired and cross-transmitted *S. aureus*

Center	acq/ct[1]	*spa* -type	MLVA-type[2]	Resistance
1	ct	t002	4	MSSA
	ct	t002	10	MSSA
	ct	t002	10	MSSA
	ct	t1889	11	MSSA
	acq	t024	22	MSSA
	acq	t015	10	MSSA
	acq	t002	44	MSSA
	ct	t015	46	MSSA
2	acq	t068	1	MSSA
	ct	t068	1	MSSA
	acq	t012	26	MSSA
	acq	u	33	MSSA
3	ct	t032	5	MRSA
	ct	t032	7	MRSA
	ct	t2577	12	MRSA
	ct	t032	13	MRSA
	acq	t032	16	MRSA
	ct	t1474	17	MRSA
	acq	t002	18	MRSA
	ct	t032	19	MRSA
	acq	t032	28	MRSA
4	acq	t002	1	MSSA
	ct	t012	3	MSSA
	acq	t008	9	MSSA
	ct	t238	4	MSSA
	acq	t032	28	MSSA
	ct	u	37	MRSA
	acq	t282	42	MSSA
	acq	t701	43	MSSA
	acq	t084	45	MSSA
	acq	t084	49	MRSA
	acq	t084	56	MRSA
	acq	t008	65	MSSA
	acq	t012	66	MSSA
	acq	t032	68	MSSA
	acq	t084	73	MSSA
	ct	t084	74	MSSA
	acq	t002	6	MRSA
	acq	t002	76	MSSA
5	acq	t645	1	MSSA
	ct	t645	1	MSSA
	acq	t012	9	MSSA
	acq	u	12	MRSA
	acq	t2155	15	MRSA
	ct	u	13	MRSA

[1] Acq/ct = acquired / cross-transmission
[2] MLVA-type is local. Identical MLVA-types from different centers are not related

Appendix II

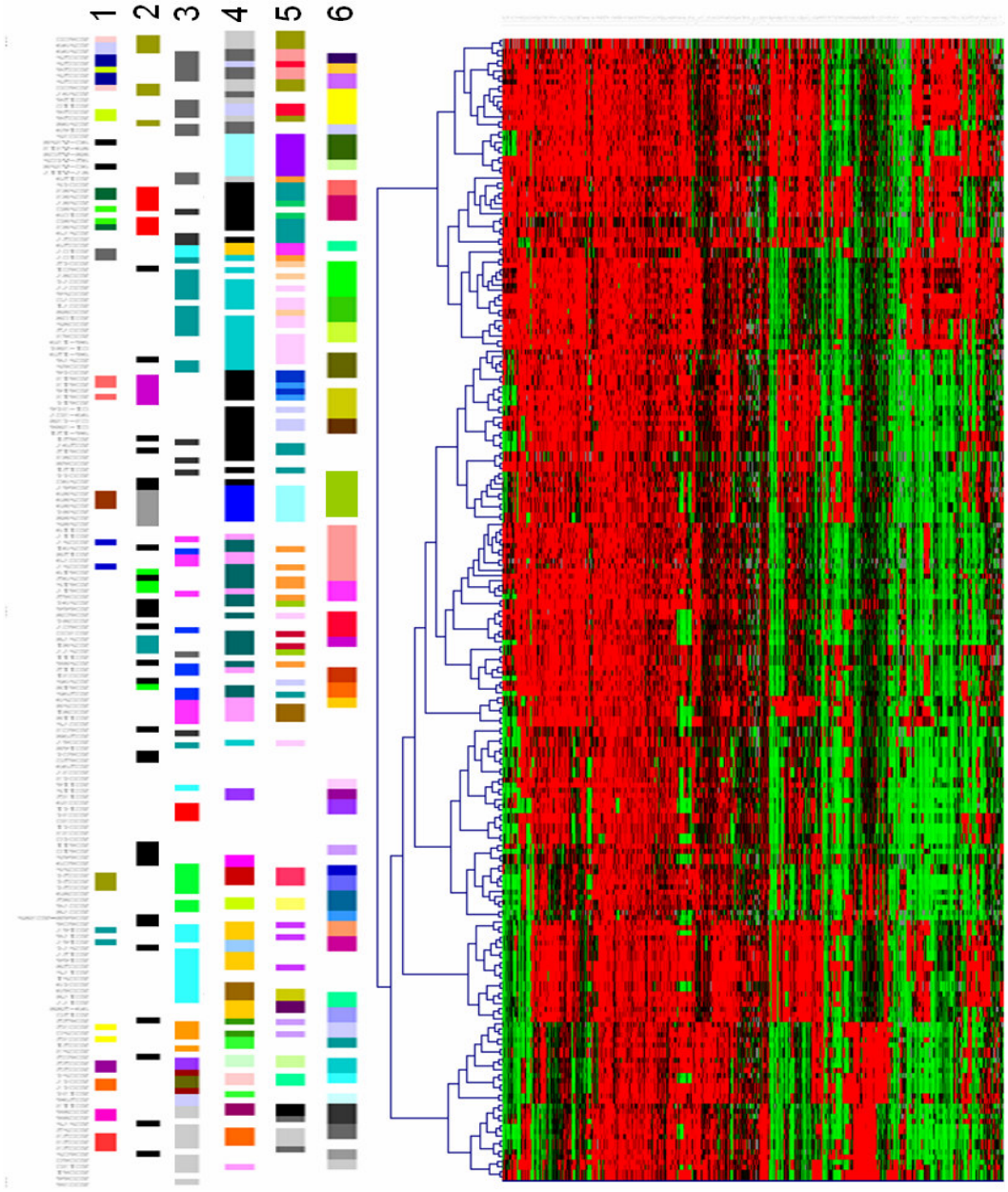

Figure 1

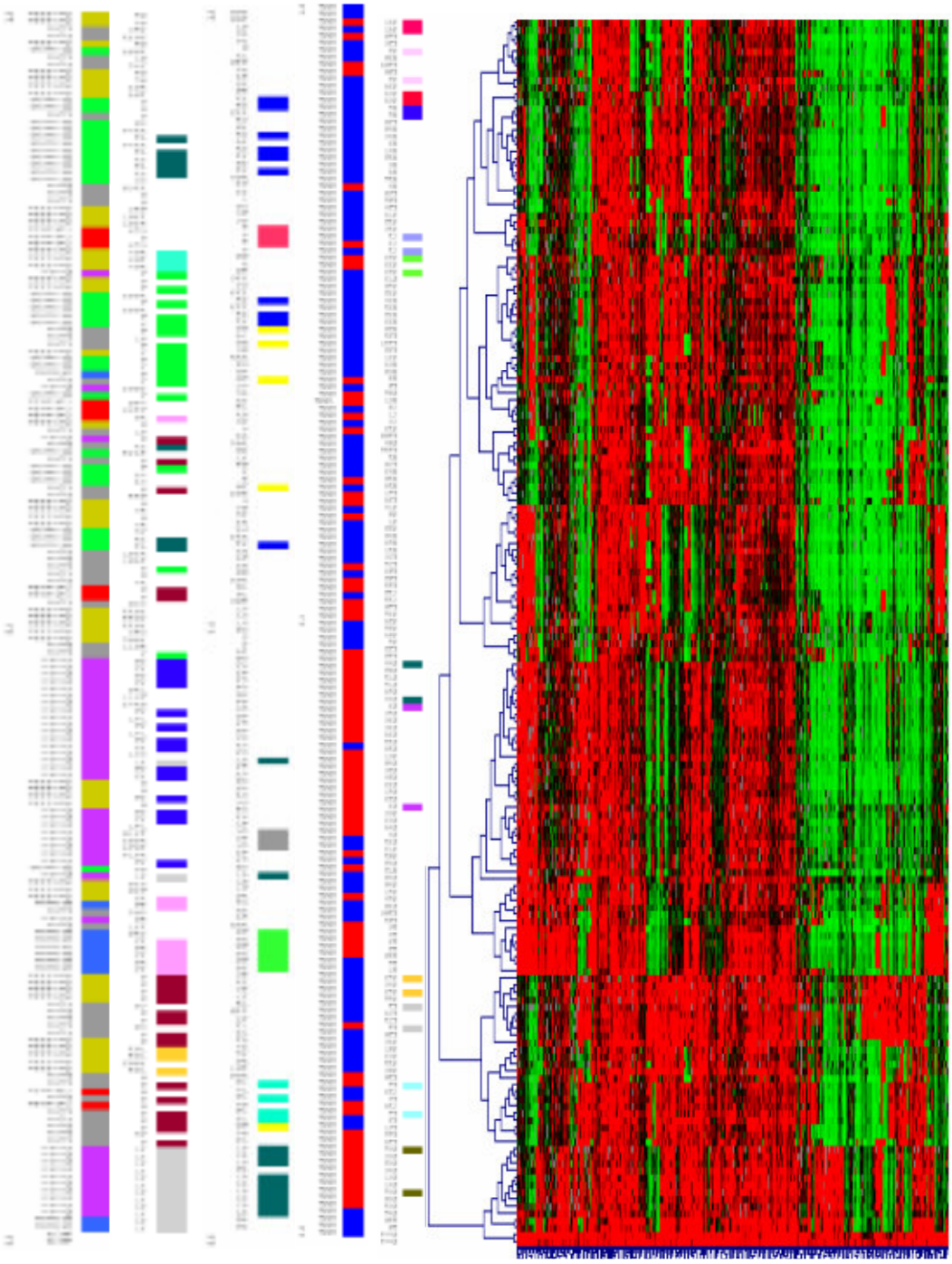

Figure 2

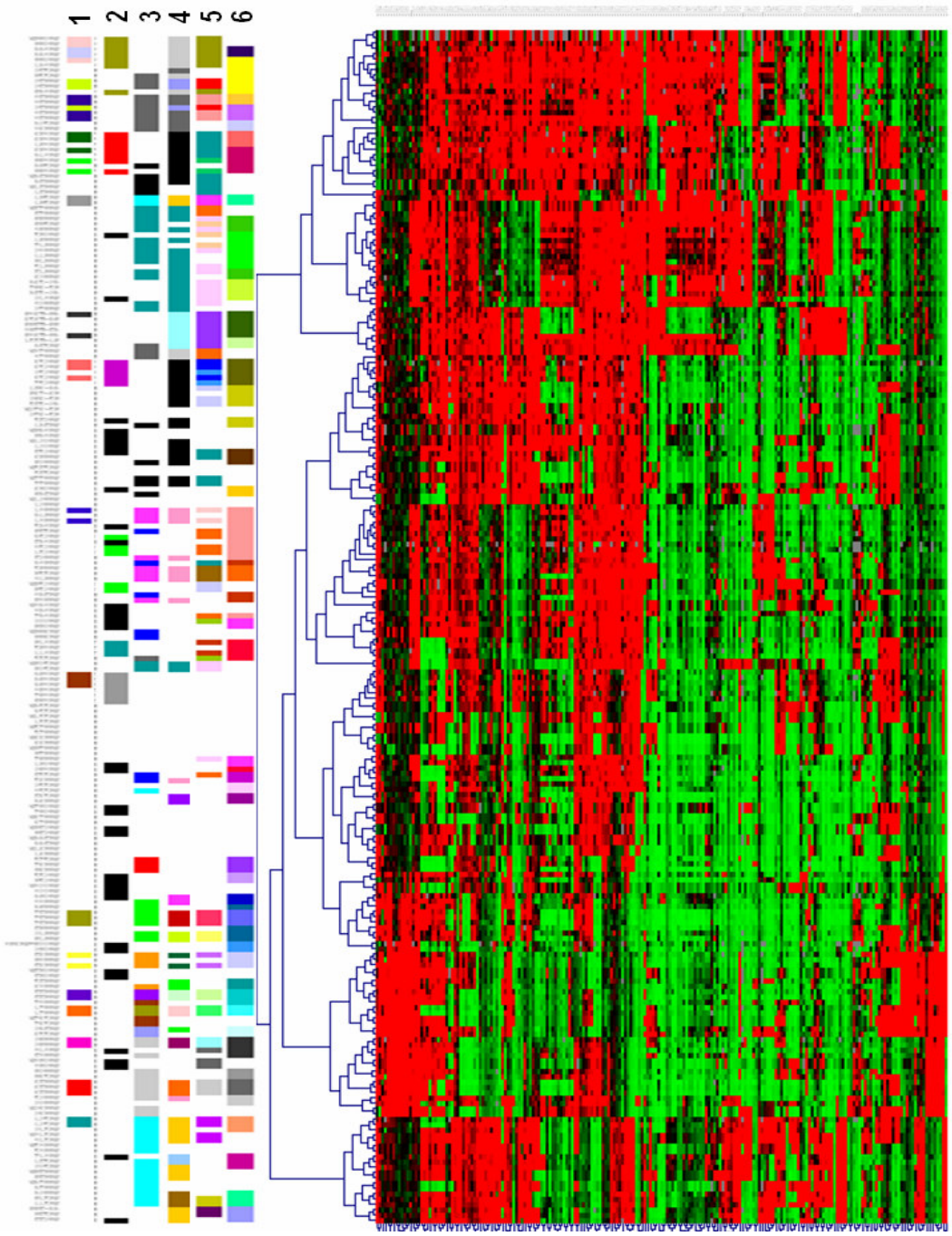

Figure 3

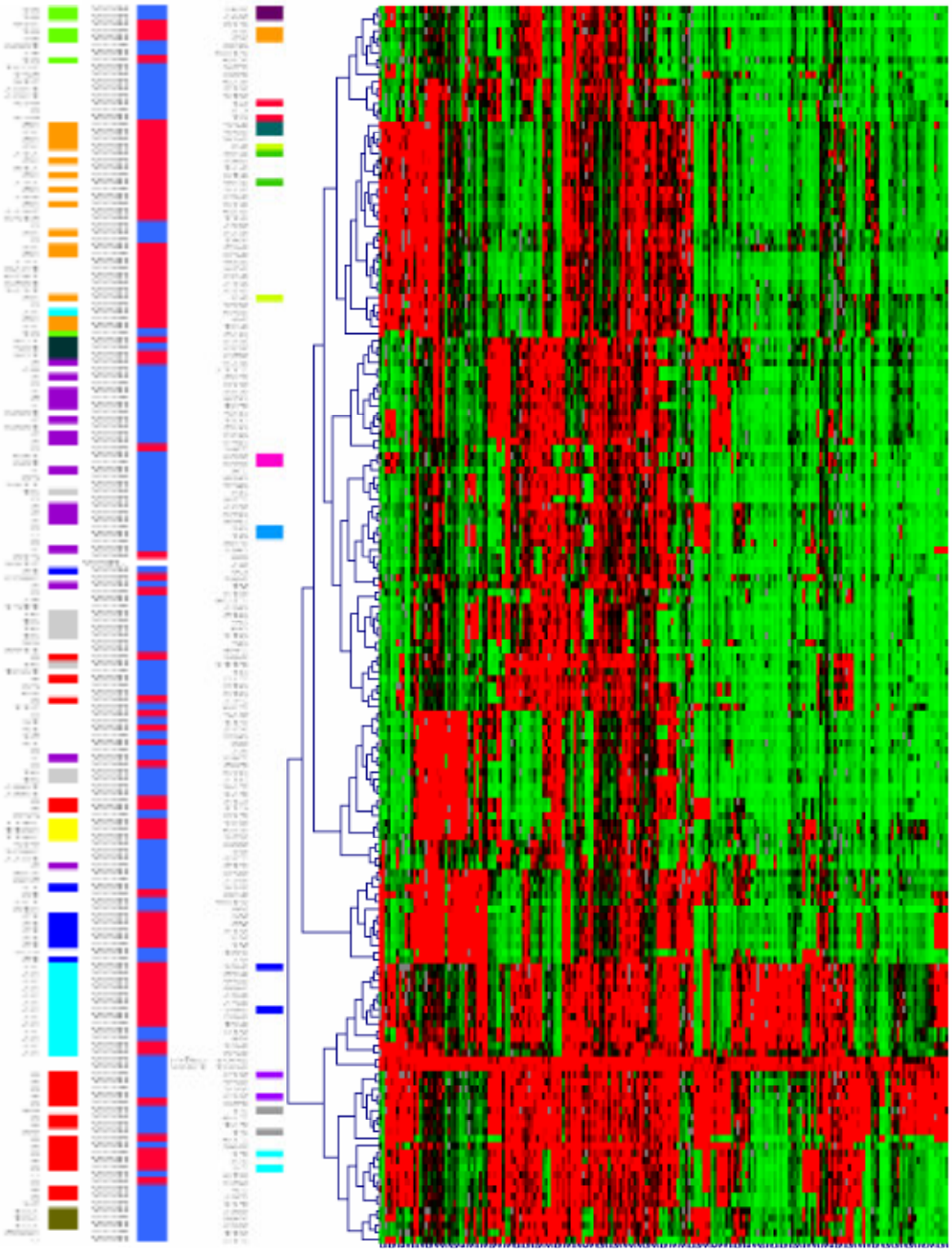

Figure 4