New insights into molecular typing methods for *Staphylococcus aureus*

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New insights into molecular typing methods for *Staphylococcus aureus*

Nieuwe inzichten in moleculaire typeringsmethoden voor *Staphylococcus aureus*

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To my family

Committee: Prof. Dr. Prof. I. M. Hoepelman (chairman)
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Chapter 1

General introduction

Introduction

Yellow-to-orange pigmented grape-like clustered bacteria were named *Staphylococcus aureus* by Rosenbach in 1884; just 3 years after Ogston initiated studying this bacterium, which was isolated from human abscesses. Rosenbach showed that *S. aureus* is a life-threatening pathogen due to its ability to cause post-operative infections and fatal pneumonia as a frequent complication of influenza (13). Ever since, *S. aureus* was classified as one among the deadliest of all disease-causing pathogens.

Humans are a natural reservoir of *S. aureus.* This bacterium is ubiquitous and may be part of human flora found in axillae, inguinal and perineal areas, and the anterior nares. Twenty percent of people are persistent carriers, 60% are intermittent carriers, and 20% hardly carry *S. aureus* (64). Asymptomatic carriage appears to play a key role in the epidemiology and pathogenesis (64). The vast majority of infections are a result of asymptomatic carriage (12,27). A wide variety of infections can be caused by *S. aureus*. The superficial skin and soft-tissue infections caused by *S. aureus*, e.g., staphylococcal scalded skin syndrome (SSSS), bullous impetigo or pemphigus neonatorum when this occurs in infants, folliculitis, furuncle, carbuncle (abscesses) that can be self-limited but may also disseminate and cause life-threatening septicemia. *S. aureus* is also causing significant numbers of deep tissue infections such as pneumonia, osteomyelitis, septic arthritis, renal and breast abscesses, endocarditis, and *S. aureus* meningitis (13,114,133). This bacterium represents a serious public health burden both in hospital and community settings.

Most infections caused by *S. aureus* can be treated by different antibiotics. The choice depends on the type of infection, e.g., flucloxacillin is frequently used to treat severe infections. In 1959, the semi-synthetic β-lactam methicillin (a progenitor of flucloxacillin) was introduced to fight infections due to penicillin-resistant *S. aureus* strains. Unfortunately, within 2 years the first *S. aureus* strain that had acquired resistance to methicillin was reported in the United Kingdom (26). This strain contains an extra DNA fragment termed *mecA* that located on a mobile genomic island called the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (18), which is absent in methicillin-susceptible strains. The *mecA* gene encodes a low-affinity penicillin-binding protein (PBP2a) that is insensitive to all β-lactams (96). Nowadays, methicillin-resistant *S. aureus* (MRSA) is not only

resistant to all β-lactams, but usually also to a wide range of other antibiotics. In addition, *mecA* has been reported contribute to the dissemination of MRSA (129).

Worldwide emergence of MRSA

MRSA has been regarded as a major nosocomial pathogen worldwide. Soon after the first MRSA was detected, new MRSA strains continued to emerge, evolve and pose a great challenge through outbreaks and dissemination within countries, cross-border and even intercontinentally (3,30). In the mid 1990s, an increasing number of outbreaks have been reported in Europe, Canada, USA, Australia, and Asia (7,32,49,94,101). Variation in the prevalence of MRSA exists between institutions and geographical areas. MRSA is rare in Scandinavian hospitals (< 2%), while it is more prevalent in hospitals in Mediterranean countries (> 40%) (110,111). The prevalence of MRSA in hospitals worldwide during 1997 – 1999 has been investigated by The SENTRY Antimicrobial Surveillance Program. They observed that the MRSA prevalence was 23% in Australia, 67% in Japan, 35% in Latin America, 40% in South America, 32% in the USA, and 26% in Europe (18,19). In addition, a north-south gradient of the MRSA prevalence has been observed in Europe (Fig.1).

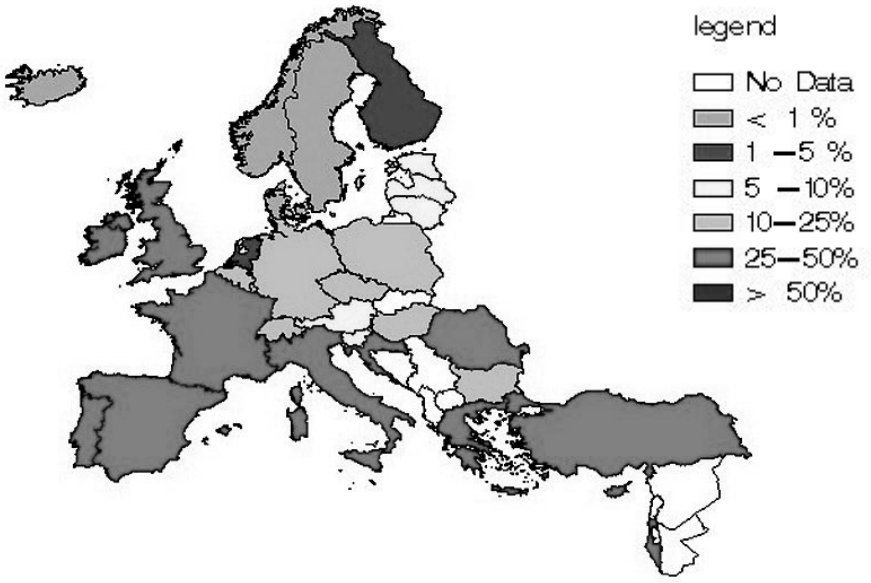

Figure 1. Proportion of MRSA isolates in 33 participating countries in Europe in 2007 reported by the European Antimicrobial Resistance Surveillance System (EARSS). (http://www.rivm.nl/earss/).

During the last 45 years, hospital-acquired MRSA (HA-MRSA) has become pandemic. Since the 1990s, the nature of MRSA started to change towards a community-associated pathogen (CA-MRSA), as the first case was reported in non-hospitalized patients in the far north of Western Australia (116).

Mobile resistance element SCC*mec*

The Staphylococcal Cassette Chromosome *mec* (SCC*mec*) is a mobile genomic island, which ranges from 21-67 kb in length and is integrated into the chromosome of MRSA near the origin of replication (28,55). The SCC*mec* element contains a set of recombinase genes, *ccrA* and *ccrB* or only *ccrC* that encode the enzyme that catalyzes the precise excision and site-specific integration (*aatBscc*) of this element (61). Several types of *ccrA* and *B* have been described. The transcription of the *mecA* gene is regulated by *mecR1-mecI*, a transmembrane β-lactam-sensing signal transducer and a repressor, respectively (96). The genetic organization around *mecA* is variable. The region is indicated as the *mec* complex. A complete *mecI-mecRI-mecA*-IS*431* is known as class A *mec*, while class B *mec* is lacking *mecI* and part of *mecR1* and it composed of *ΔmecR1-mecA*-IS*431*. At least 8 different *mec* complexes have been described, depending on the IS element present, its orientation and the nature of the deletion in *mecR/I*.

Insertion element IS*431* located downstream of *mecA* acts as a receptor site for integration of IS*431*-associated plasmids and transposons which lead to the accumulation of resistance determinants (96). So, SCC*mec* has various gene compositions in different strains (29). Table 1 shows genetic determinants which may or may not be present in SCC*mec* (30,51,86). At the moment, there are seven main types of SCC*mec* (18,50) which have been determined by the *ccr* and *mec* complex (Figure 2). The regions that are not part of the *mec* complex and *ccr* genes are called J (junkyard) regions (15,51,54,62,86).

Table 1. Genetic determinants which may present in SCC*mec*.

Determinant	Functions
mecA	Methicillin resistance
mecI	Repressor for *mec*A promotor
mecR1	Receptor for β-lactam antibiotics in *mecA* regulation
ccrA	Cassette chromosome recombinase A, involved in SCC*mec* excision from and integration into chromosome
ccrB	Cassette chromosome recombinase B, involved in SCC*mec* excision from and integration into chromosome
ccrC	Cassette chromosome recombinase C, involved in SCC*mec* excision from and integration into chromosome
Tn*554*	Encodes erythromycin and spectinomycin resistance and transposase genes for Tn*554*
Pseudo Tn*554*	Cadmium resistance and transposase gene Tn*554*
IS*431*	Transposase genes for IS*431*
pUB110	Integrated plasmid encodes tobramycin and bleomycin resistance and contains *pre* gene encoding pUB110 recombinase
pT181	Encodes tetracycline resistance
pI258	Resistance against penicillins and heavy metals
IS*256*	Transposase
IS*257*	Transposase
PseudoIS*1272*	Transposase gene IS*1272*

The epidemiology of MRSA

The occurrence of a new type of SCC*mec* corresponded to a new wave of outbreaks. The first MRSA isolated from UK in 1961 known as the Archaic clone, which disseminated worldwide and dominated until the 1970s, was associated with the SCC*mec* type I (14,22). The second wave of MRSA, in the early 1980s, was first reported in Japan and characterized by the SCC*mec* type II. This clone was called the New York/Japan clone (18). An MRSA harboring SCC*mec* type III was first found in New Zealand in the late 1980s and marked the third wave of MRSA (18,30). These three SCC*mec* types were strongly associated with the hospital-acquired MRSA (HA-MRSA) infections (30). Then SCC*mec* type IV was characterized. The first isolates with SCC*mec* type V and VI were isolated in Australia and Portugal, respectively. Only type IV and V disseminated worldwide. SCC*mec* type IV and V are predominant in community-associated MRSA (CA-MRSA) (30,82). SCC*mec* type VII was recently discovered in The Netherlands and Taiwan (18,50).

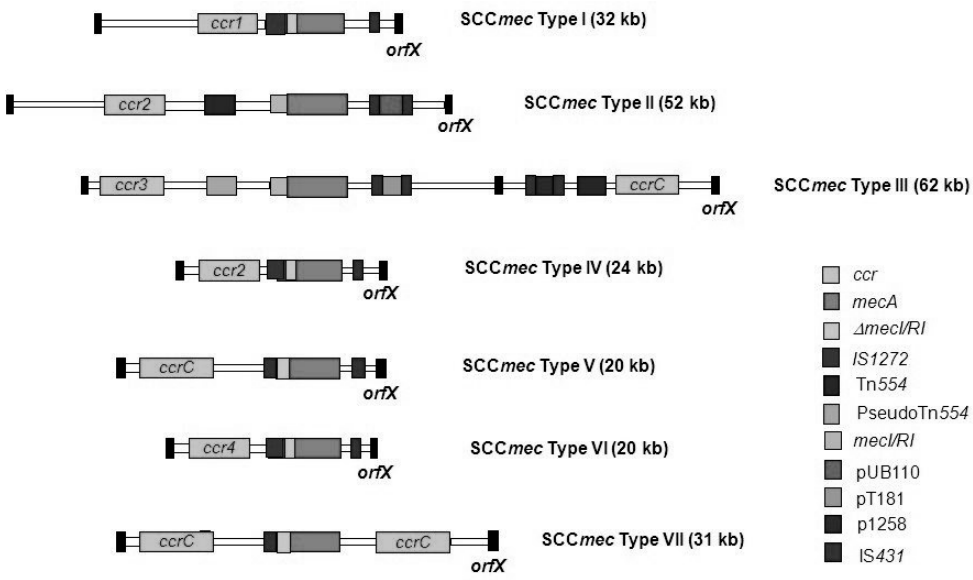

Figure 2. A schematic drawing of the seven SCC*mec* types (I-VII). All the SCC*mec* cassettes are integrated in a conserved open reading frame (*orfX*). Colored boxes represent the major elements of each SCC*mec* cassette.

Emergence of MRSA in the animal reservoir

During the last few decades, MRSA has emerged in the animal population as indicated by the increasing numbers of reports documenting the occurrence of MRSA in animals, such as domestic pets, horse, sheep, poultry, bovines and pigs (120,123-126,131). These isolates were commonly believed to originate from humans. However, since 2004 MRSA ST398 is widespread among pigs. This ST was found among pigs and pig-farmers (17). Before 2004 ST398 was only described among 4 pigs and 2 pig-farmers in France, but all isolates except one isolate from a farmer were MSSA (6). A ST398 human clinical methicillin-susceptible *S. aureus* (MSSA) isolate from the Cape Verde islands (CV55) has been detected. But whether this isolate was pig- related is not known (1). ST398 MRSA can be transmitted from pigs to humans (69,125). Although transmission is mainly between animals, contact with pigs has been identified as a risk factor for MRSA ST398 carriage (6,63,120,121,125,130). ST398 MRSA can cause invasive disease in humans as demonstrated for example by a case of endocarditis(24). ST398 MRSA was isolated from pigs that suffered from exudative epidermitis and dairy cattle with mastitis (24,121) Studies in The Netherlands showed that more than 20% of the pig farmers and 39% of slaughterhouse pigs carried MRSA which

belonged to ST398 (17,131). This also has been described in other countries such as Canada, North America, France, Denmark, and Singapore (41,68,69,103). Almost all isolates associated with ST398 belong to the *spa*-type t011, t034, t108, t567, t899 and t939 (9,79,121). These *spa*-types appear to be related. ST398 isolates harbor SCC*mec* type IV or VII (69,119).

ST398 MRSA has also been described in poultry and bovines, especially calves. However, MSSA have become the number one pathogen causing bovine intramammary infection (IMI) known as bovine mastitis. Both subclinical and clinical mastitis cause economic losses in the dairy industry due to reduction in milk production and culling or death of infected animals, while chronic and deep infections in the mammary glands of bovines are often associated with poor success of treatment (11,40,48,59,108,127). The first MRSA isolated from a bovine was reported in the 1970s which would not be surprising due to the widespread usage of intramammary antibiotics (68), but MRSA infections are still relatively rare. Only a few molecular studies have explored the population structure and genetic relationships of *S. aureus* from bovine mastitis. A previous study suggested that a small number of clonal types are responsible for widespread occurrence of mastitis (59). Others have shown a difference between the population of human and bovine *S. aureus* and some of them suggested host-specificity among *S. aureus* clones (39,59,93,106). Although one study could not demonstrate host specificity (2).

The pathogenesis of *S. aureus* in the bovine udder is not fully understood. But it is believed that a combination of secreted staphylococcal enterotoxins is the most important contributor to the pathogenesis (48).

Virulence factors of *S. aureus*

Successful colonization of both humans and animals by *S. aureus* depends on the combination of the host defense mechanisms, genetic predisposition of the host, and virulence factors produced by *S. aureus*. A wide spectrum of secreted and cell-surface associated virulence factors can be expressed to promote adhesion to the host extracellular matrix components, and host-cell damage (33). These virulence determinants are thought to contribute the invasiveness of *S. aureus*, although no single strain encodes all the known *S. aureus* virulence factors.

A large number of staphylococcal enterotoxins (SEs) have been recognized, including the five classical enterotoxins: SEA to SEE, and the recently characterized SE types SEG to SEU (4,21,107,132). *S. aureus* may secret toxic shock syndrome toxin 1 (TSST-1) which is known as the first toxin causing toxic shock syndrome (21), a cytotoxic protein with hemolysin activity named β-hemolysin (sphingomyelinase C) encoded by *hlb* (21), the bi-component Panton-Valentine Leukocidin (Luk-PVL) which may destruct leukocytes and causes tissue necrosis that makes this protein mostly associated with furuncles and severe necrotizing pneumonia (74,80), and exfoliative toxin A and B (ETA and ETB) with protease activity and the ability to induce T-cell proliferation (78). The biological activity of *eta* and *etb* to induce intra-epidermal skin peeling, is mostly associated with staphylococcal scalded skin syndrome (SSSS) and bullous impetigo (43,78). Combination of one or more virulence associated genes such as the SE and the TSST-1 gene is often observed in 10 to 200 kb mobile genetic elements, termed *S. aureus* pathogenicity islands (SaPI) (70).

Adhesion of *S. aureus* to the host extra-cellular matrix components, such as fibrinogen, fibronectin and collagen is promoted by cell surface adherence proteins that belong to the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (33). Clumping factor A and B (ClfA and ClfB) mediate binding to fibrinogen (33,89,128), fibronectin-binding protein A and B (FnBPA and FnBPB) mediate binding to fibronectin, while *cna* produces a collagen adhesive protein (81). Serine-aspartate repeat proteins (Sdr) are surface proteins related to ClfA and ClfB and have been hypothesized mediate interactions of *S aureus* with the extra-cellular matrix (38,58,100). Bone sialoprotein-binding protein (BSP) is a member of the Sdr family and has been speculated to contribute to osteomyelitis (115). Elastin-binding protein of *S. aureus* (EbpS) is an unique integral membrane protein expressed on the cell surface and mediates binding to soluble tropoelastin (23). The polymerization of fibrinogen to fibrin is induced by coagulase, while fibrinogen-binding protein (Efb) is an intracellular protein which specifically promotes binding to fibrinogen (10). To be able to colonize different tissues, adherence of *S. aureus* is enhanced by a MHC class II analog protein (MAP), a protein with broad binding activity to fibrinogen, collagen, fibronectin, vitronectin, and elastin (67,88). Plasmin-sensitive protein (Pls) has adhesive and anti-adhesive functions at a certain stage during infection. This protein mediates interaction between bacterial cells and also allows bacteria to spread (57,102). Polysaccharide intercellular adhesin (PIA) synthesized by the gene products of *icaADBC* locus is necessary for biofilm

formation (65,83,95). Studies have reported the presence of *icaADBC* nearly in all *S. aureus* strains (34,65,90,95).

The ability of *S. aureus* to escape the human immune system is provided by phages encoding immune evasion molecules such as chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS, staphylococcal complement inhibitor (SCIN) and staphylokinase (SAK) (16,97).

Other *S. aureus* proteins which also contribute to virulence include serine protease (SspA) and cysteine protease (SspB) with elastinolytic activity for elastin degradation (60,91) and capsular polysaccharide (CP) which renders the bacteria resistant to phagocytosis (84,92,109). In addition, the colonization and virulence of some *S. aureus* strains is also enhanced by the presence of an arginine catabolic mobile element (ACME) which encode protein that inhibit polymorphonuclear cell production (20). However, while the virulence factors in human S. *aureus* isolates have been extensively studied the presence of virulence genes in animal associated *S. aureus* have been identified on a limited scale only.

Typing methods for *S. aureus*

For more than 30 years, typing techniques have been progressing rapidly. They have been applied to elucidate the basic mechanisms of pathogenicity and to track the spread of pathogens (122). Several typing methods have been developed to speed and improve characterization of MRSA. These methods can be divided in phenotypic and genotypic methods.

Phenotypic methods identify the bacteria on the gene expression level. These methods employ all phenotypic properties such as biochemical profiles, susceptibility to bacteriophages, antigens present on the cell's surface, whole protein analysis and antimicrobial susceptibility patterns. But since gene expression is highly influenced by the environment during culture, all phenotypic typing methods have a tendency to vary, thereby limiting reproducibility and reliability of these methods (122).

Genotypic methods involve DNA-based technologies that have been introduced and developed in the last two decades. These technique have been increasingly

used in clinical laboratories including “band-based” and “sequencing-based” methods (18).

At present, the molecular genotyping methods are commonly used for *S. aureus* and include pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST), *spa*-typing, multiple locus variable number tandem repeat (VNTR) analysis (MLVA), and SCC*mec* typing (the latter only for MRSA) (8,18,25,35,36,46,52,112,113,117).

Pulsed-field gel electrophoresis (PFGE)

PFGE has been considered a gold standard since it gives high discriminatory power. For *S. aureus*, the chromosomal DNA is digested with the restriction enzyme *SmaI* resulting in 10-20 DNA fragments, that are separated on agarose gel. Analysis of the result is based on the obtained banding patterns (112). It should be noted that pig-associated MRSA strains were nontypeable by PFGE using *SmaI* due to the presence of a 5’-methylcytosine at specific sites in its recognition sequence, CCCGGG, which prevents digestion by the restriction enzyme (9). However, PFGE is laborious, relatively difficult to perform and slow. Furthermore, interlaboratory data comparison is difficult to achieve.

Amplified fragment length polymorphism (AFLP)

If data exchange is a hurdle for PFGE, AFLP is more suited for inter-laboratory data comparison and shown to be more reproducible than PFGE. This method is an image-based DNA fingerprinting technique, which compares DNA fragment patterns generated by restriction enzyme digestion in combination with PCR amplification (75). The discriminatory power of AFLP is higher than for MLST and similar to that of PFGE.

Multilocus sequence typing (MLST)

Since DNA sequencing became widely used and more affordable, MLST has been proven to be a method of choice for studying the molecular evolution of *S. aureus* (26). This method is based on the allelic profile of seven house-keeping genes of approximately 500bp in length as listed in Table 2 (18, 25). The results of MLST are presented as an allelic profile that is designated a sequence type (ST), e.g., an *S. aureus* isolate with allele profile 3-35-19-2-20-26-39 belongs to ST398. An isolate with one locus different is considered as a single locus variant. Related STs are combined in clonal complexes (CCs), e.g., such as ST239,

ST247 and ST8 belong to CC8. Five major CCs of *S. aureus* have been described, CC5, CC8, CC22, CC30 and CC45 (26).

The MLST data can be replicated anywhere in the world and it offers the ease of data exchange since the results are DNA sequences. Furthermore, there is full access to a database (www.mlst.net). However, the discriminatory power of the MLST is lower than that of PFGE and therefore this method is not suitable for routine surveillance of MRSA. In addition, it is costly and access to a DNA sequencing facility is necessary (46).

Table 2. Seven housekeeping genes for MLST of *S. aureus* (25).

Gene	**Sequence length (bp)**
Carbamate kinase (*arcC*)	456
Shikimate dehydrogenase (*aroE*)	456
Glycerol kinase (*glpF*)	465
Guanylate kinase (*gmk*)	429
Phosphatase acetyltransferase (*pta*)	474
Triosephosphatase isomerase (*tpi*)	402
Acetyl coenzyme A acetyltransferase (*yqiL*)	516

Staphylococcus protein A (spa)-typing

Another DNA sequence-based typing method is *spa*-typing. This method involves sequencing of a hypervariable short sequence repeat (SSR) locus of the polymorphic X region of the protein A gene (*spa*) flanked by well-conserved regions (42,98). This locus consists of a variable number of 24 nucleotides long repeat units. The repeat units themselves also have sequence variation (105). The determination of *spa*-types was simplified by the Ridom StaphType software package (Ridom GmBH, Würzburg, Germany) (46) and full accessibility to *spa*-types database is available at www.SpaServer.ridom.de. Since *spa*-typing utilizes a single locus, this method is less expensive, less laborious and less time consuming than the MLST. *Spa*-typing has been shown to have discriminatory power between PFGE and MLST (72). Similar to the MLST, *spa*-typing is also limited by access to a DNA sequencing facility.

Multiple locus variable number tandem repeat (VNTR) analysis (MLVA)

A variable number tandem repeat (VNTR) is a short nucleotide sequence organized as a tandem repeat on the DNA chromosome. The VNTR has become essential since it has been proven to be a powerful tool in the determination of evolutionary relationships and population genetics of bacteria (45). The use of VNTRs has been increasing in the past few years as a means of typing for a number of pathogens, such as *Bacillus anthracis, Yersinia pestis, Mycobacterium tuberculosis, Legionella pneumophila, Pseudomonas aeruginosa, Salmonella enterica, Escherichia coli, Enterococcus faecium*, *Staphylococcus epidermidis* and *Staphylococcus aureus* (5,45,47,56,71,73,118). In the majority of cases, the objective behind development of the VNTR-based typing, termed MLVA, is to meet the need for a fast and reliable typing method for outbreak situations (71).

The MLVA schemes are based on direct PCR amplification of specific loci with VNTRs. So, unlike the “anonymous” bands as in PFGE or AFLP, all targets in MLVA are known (71). The number of repeat units (RU) varies from strain to strain (52,99). The variability of RU may be explained by slipped strand mis-pairing (118), a deletion/insertion process during DNA replication which involves denaturation and displacement of DNA strands, resulting in mispairing of the complementary bases. This mechanism is possibly only involved for small repeat units, whereas recombination is more likely the mechanism of variation in repeat number for large repeat units. In addition, it has been hypothesized that the repeat variability may influence the biological function and offers evolutionary advantages, but its mechanism is unclear (118).

The first MLVA for typing scheme for *S. aureus* has been previously described and has targeted *sdr, clfA, clfB, ssp* and *spa*. It involved multiplex PCR and visualized its product as a banding pattern on standard agarose gel (99). Thus, bands could not be assigned to individual loci and the RU of each locus could not be conveniently calculated. This fact is considered a drawback of the first MLVA scheme for *S. aureus*.

Several VNTRs of *S. aureus* have been identified and termed as Staphylococcal Interspersed Repeat Units (SIRUs). Seven of them have been used for typing (45). The seven SIRUs are SIRU01, SIRU05, SIRU07, SIRU13, SIRU15, SIRU16, and SIRU21, which are found to be distributed around the genome of seven sequenced *S. aureus*, COL, MW2, Mu50, NCTC 8325, MRSA 252, MSSA 476, and N315 (Fig.3) (45).

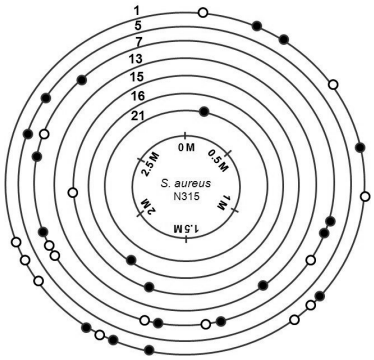

Figure 3. Distribution of SIRU on the genome of MRSA strain N315. The position of each SIRU is represented by black and white dots. The black dots depicted positive DNA strands while the white dots represented negative DNA strands. Number of bases around the genome is shown in the centre circle with 0M as the origin of replication (45).

SIRU13, 15, 16, and 21 occur only once on the genome, while SIRU01, 05 and 07 are multiple-site SIRUs. The latest three SIRUs show little similarity in flanking regions within the same genome. Only SIRUs with flanking sites that contained the highest sequence homology between genomes were chosen for analysis. Six SIRUs are in non-coding regions. SIRU13 for example is located upstream of *hmrB*, a gene involved in the conversion of heterogeneous to homogeneous expression of methicillin resistance (66). SIRU21 is in the coding region of *spa* (45). The length of the repeats ranges from 24 – 159 bp (44), and variations of RU within the SIRU are observed (45). However, function of SIRUs is not known.

Microarray

DNA microarrays have recently been described as a powerful genotyping technique because they can simultaneously detect thousands of genes or target DNA sequences on a single glass slide. This method is based on the principle of hybridization (31) and target (probes) can be either PCR products or short oligonucleotides based on the available genome sequences (37). DNA microarrays detect the presence or divergence of the target by evaluating the differential signal produced by the hybridization products. The microarray system is expected to be a powerful epidemiological tool (87), but the use of this technology is still very sporadic due to cost. However, future applications could use selected regions of variability in whole genome arrays as standard procedure in epidemiologic investigations (104).

SCC*mec* typing

A method to exclusively characterize the MRSA strains and particularly the mobile element carrying *mecA* has been developed and termed SCC*mec* typing. Two different approaches have been developed for molecular characterization of the mobile genetic element carrying *mecA* (SCC*mec*). The first strategy uses a multiplex PCR to detect *mecA* and different loci within the junkyard (J1) region of SCC*mec* type I to IV (85). Recently, this has been updated to include SCC*mec* type V and VI (76,77). However, this method does not include the determination of the *ccr* genes which is sometimes needed for the correct classification of SCC*mec* (18). A second strategy applies several PCR assays to detect the structure of *mec* complex and type of *ccr* genes (53).

Outline of the thesis

The MRSA problem has been increasing at an alarming speed and recently MRSA has emerged and spread in the animal reservoir. Various typing methods that are currently available give insight into the epidemiology of the MRSA infection. Nevertheless, development of high resolution typing methods is needed to improve the investigation and control outbreaks. In addition, the methods need to be fast and reliable. The main aim of the thesis was the development of a typing method for the detection of *S. aureus* including MRSA in outbreak situations in different reservoirs. In addition, we wanted to get more insight in the spread of *S. aureus* in the animal reservoir.

The following research questions were addressed:

1. Can we develop and validate a rapid and reliable typing tool for human *S. aureus*, particularly in case of outbreaks that is not based on banding patterns or DNA sequencing?

2. Is it possible to apply the newly developed typing scheme to animal reservoirs of *S. aureus*?

3. What is the position of bovine *S. aureus* in the population structure of *S. aureus* as obtained by MLST? What is the relationship with human-derived *S. aureus*?

4. What is distribution of virulence factors in genotyped *S. aureus* from bovine mastitis, that possibly may underlie the mastitis pathogenesis?

5. Do different kinds of pig farms play a role in transmission of MRSA?

6. Can microarray technology with a limited selection of DNA probes be used for typing of outbreaks of human *S. aureus*?

To address these issues we evaluated and developed a novel MLVA system for human clinical S. *aureus* isolates (**Chapter 2**), and extended the use of this MLVA system to animal reservoir (bovine mastitis MSSA and pig-related MRSA) as described in **Chapter 3 and 4**. In these 3 chapters, the performance of the MLVA scheme was evaluated for typeablity, reproducibility, stability, discriminatory power, and epidemiologic concordance. **Chapter 5** described the characterization of genotyped Dutch bovine mastitis MSSA by means of identification of the virulence factors genes. In **Chapter 6** we demonstrate and discuss reasons for the transmission of MRSA between pig farms. In **Chapter 7**, we evaluated the use of microarray using selected probes on human clinical *S. aureus*, which was further validated with the isolates collected in six ICUs across Europe.

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Chapter 2

A novel multiple locus variable number tandem repeat (VNTR) analysis for rapid molecular typing of human *Staphylococcus aureus*

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ABSTRACT

The objective was to develop and evaluate a Multiple Locus Variable-number tandem repeat Analysis (MLVA) typing system that can be combined with *spa*-typing for human *Staphylococcus aureus*. PCR was performed on 6 Staphylococcal Interspersed Repeat Unit (SIRU) of 100 European clinical isolates, which were well-distributed among the *S .aureus* population based upon Multi-Locus Sequence Typing (MLST). The size of the repeat units (RU) of SIRU vary from 24 to 131 bp. The number of RU was calculated for each SIRU and each unique combination of the numbers of repeats was assigned an MLVA-type (MT). Variations in RU were observed in all loci. Comparison of MLVA with MLST and *spa*-typing revealed that among 100 *S. aureus* isolates 71 MLVA-types, 32 Sequence Types, and 47 *spa*-types could be distinguished. The Simpson's Indexes of diversity for MLST, *spa*-typing and MLVA were 0.941, 0.963 and 0.987, respectively, indicating that MLVA displays the highest discriminatory power. The Adjusted Rand index and Wallace's coefficient indicated that MLVA was reasonably well predictive for both MLST and *spa*-type, but not vice versa. For validation 50 isolates were used, representing isolates belonging to 6 outbreaks (n= 22) and 28 isolates without epidemiological link. In general, all epidemiologically related isolates had the same MLVA-type, while unrelated isolates had different MLVA types. The Wallace's coefficients for this set of isolates indicated that MLVA was highly predictive for *spa*-type. The typing of 14 isolates collected over 2-7 years from 4 patients showed that MT are stable over at least 5 years. The MLVA typing method described here is a rapid and simple method for typing MRSA for epidemiological purposes.

INTRODUCTION

Staphylococcus aureus remains a significant problem causing infections in both hospital and community setting. Methicillin-resistant *S. aureus* (MRSA) continue to emerge and evolve and pose a great challenge through outbreaks within countries, cross-border and even pandemic spread (6). Early recognition and detection of pathogens which may cause an outbreak is important for infection control. Much effort has been put in the development of molecular typing methods to speed up and improve the characterization of methicillin-resistant *Staphylococcus aureus*. The history of typing of *S. aureus* started with the introduction of bacteriophage typing. It has been used for more than 30 years since the 1960's. Unfortunately, this method had poor reproducibility and low typeability, and moreover accessibility of this method was limited to only a few reference laboratories that had a large number of phage stocks and propagating strains. Bacteriophage typing was replaced by Pulsed Field Gel Electrophoresis (PFGE) which has been considered as a gold standard method since it has high discriminatory power, but this technique is laborious, relatively difficult to perform and slow. Furthermore, interlaboratory data exchange is difficult (2,25). Recently, DNA sequence technology became more widely available and affordable. Therefore, sequence-based typing methods have been developed. These techniques are especially used in research laboratories with easy access to sequencing facilities (5). Enright *et. al*. introduced a method to characterize strains of *S. aureus* based on allelic profile of the seven house-keeping genes known as a Multi Locus Sequence Typing (MLST). The results of MLST are in digital format and full access to a database (www.mlst.net) offers the ease of data exchange to all MLST users worldwide. The MLST database currently contains information of more than 1800 isolates from 50 different countries (3,4). However, the discriminatory power of the MLST is lower than that of PFGE and sequencing of DNA fragments of 7 genes is expensive. Another relatively new method is *spa*-typing which is based on sequence variations in a region of Protein A of *S. aureus* (*spa*) having 24 nucleotides long repeat units. Full accessibility to a database is also available (www.SpaServer.ridom.de) which currently covered more than 4800 isolates from 47 countries. This method is less expensive than MLST, but *spa*-typing is also limited to the availability of DNA sequencing (7,11,23). So, all these typing methods have a number of limitations for routine application in less well equipped laboratories.

In the past few years Multiple Locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA) has become popular and has been successfully used for typing of a number of pathogens (1,12,15,17,28). Repetitive DNA sequences or so-called VNTR are present in the chromosomes of both prokaryotes and eukaryotes. They have a unique length and DNA sequences that may vary between species (10,28). The first MLVA for *S. aureus* described by Sabat *et. al.* targeted the *clfA*, *clfB*, *sdr*, *spa*, and *sspA* genes and the results were comparable to the PFGE (22). This finding was supported by other investigators (8,16,26). Hardy *et. al*. described an MLVA scheme based on seven VNTRs in *S. aureus* termed Staphylococcal Interspersed Repeat Units (SIRUs) which were distributed on either single or multiple sites with specific flanking regions on the genome. SIRU01, 05, 07, 15 and 16 were in non-coding regions, while SIRU13 and 21 were located in the coding region for a hypothetical protein MW1681 and Protein A (*spa*), respectively (9,10). The major drawback of these MLVA schemes is that they are fingerprint methods, which makes interlaboratory comparisons more difficult and require specialized equipment for analysis.

In this study, we aimed to develop and evaluate a MLVA system for human *S. aureus* with analysis on agarose gel based on reporting the number of repeats for each locus and that can be used in combination with *spa*-typing.

MATERIALS AND METHODS

Bacterial strains

One hundred human *Staphylococcus aureus* isolates were selected from the collection of the European Network for Antibiotic Resistance and Epidemiology (ENARE) at the University Medical Centre Utrecht in The Netherlands. Selection of the bacterial strains was based on MLST data obtained previously. MLST was performed according to Enright *et. al* (4) and was intended to include isolates that represented various sequence types (STs). The 100 isolates represented 35 MLST types that 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 (Figure 1). These 100 samples were clinical isolates of which 25 *S. aureus* were susceptible to methicillin (MSSA) and 75 were MRSA. The isolates were collected between 1997 and 2004.

Fifty clinical methicillin-resistant *S. aureus* isolates collected by the Department of Infection Control and Infection Prevention of the University Medical Centre Utrecht, The Netherlands were used in the second part of this study for validation of the findings with the ENARE isolates. Twenty two isolates belonged to 6 outbreaks, while 28 isolates originated from the Netherlands and were epidemiologically unlinked. The origin of these isolates was hidden from the researcher before and during the experiments.

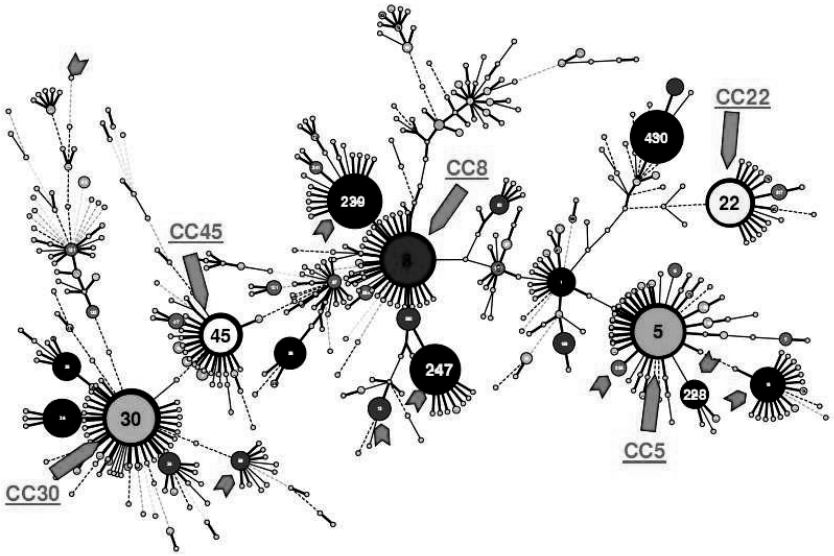

Figure 1. Minimum spanning tree of the *S. aureus* population structure based on MLST. Each circle represents a different ST. The number in the circle indicates the ST. Orange arrow indicates the distribution of samples used in this study. Thus it is obvious that they were well distributed, represented the five major clonal complexes (CC5, CC8, CC22, CC30 and CC45) and singletons.

In addition, 14 isolates from the Department of Infection Control and Infection Prevention of the University Medical Centre Utrecht, The Netherlands collected from 4 patients at different time-points were included in order to study the stability of the MLVA type in time.

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. Purified DNA was measured with a NanoDrop spectrophotometer for its DNA concentration and stored at -20°C prior to use.

MLVA typing

MLVA typing performed in this study used a combination of loci (SIRU01, -05, -07, -13, -15, -16, -21 (*spa*), and *sspa*) from previous studies performed by Hardy *et. al*., and Sabat *et. al*.. The size of the repeats for each locus is shown in Table 1 (9,22). PCR was performed in 25 µl containing HotStarTaq Master Mix (QIAGEN) or SuperTaq (HT Biotech) and 10 pmol/ml of each primer. The amplifications were carried out in a GeneAmp PCR System 2700 Thermocycler (Applied Biosystems) with the following cycling parameters: 15 min initial denaturation at 95°C, 28 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 30 seconds, and in a final step the PCR product was elongated at 72°C for 7 min. Then, 1 µl of each PCR product (SIRU01, -05, -07, -13 and -15) was analyzed on a 2% agarose gel, while the SIRU21 PCR product was ran on a 3% agarose gel. A 100 bp DNA ladder (Invitrogen) was used as a size standard and loaded into the first and the last well. The DNA fragments were stained with ethidium bromide and visualized under UV-light and photographed.

Assignment of MLVA type (MT)

The number of repeats for each locus was determined by subtracting the size of the flanking regions from the size of the amplicon followed by division by size of the repeat (Table 1). The repeat number obtained was rounded to the nearest integer value. After calculating the number of repeat units (RU) of all loci, a number string was obtained as a result from the combination of RU from SIRU01, -05, -07, -13, -15 and -21. The number string was considered an allelic profile. For each allelic profile an MLVA type (MT) was assigned.

***Spa*-typing**

The *S. aureus* protein A gene (*spa*) was amplified and sequenced using a primer set as described by Harmsen *et. al*. (11). The resulting PCR product was sequenced using BigDye terminator version 3.1 on an ABI 3100 sequencer (Applied Biosystem). Bionumerics was used to analyze the obtained sequences

and to assign the *spa*-types. Novel *spa*-types were submitted on-line to the Ridom SpaServer database (www.SpaServer.ridom.de).

Pulsed-Field Gel Electrophoresis (PFGE)

Fifty MRSA isolates from the Department of Infection Control and Infection Prevention of the University Medical Centre Utrecht, The Netherlands and 14 isolates from 4 patients were genotyped by *Sma*I-generated restriction fragment length polymorphism of whole-genome DNA as described previously (25).

Tools for comparison of MLVA, *spa*-typing, MLST and PFGE

The MLVA types were clustered using the minimum spanning tree algorithm available in Bionumerics software (version 4.5; Applied Maths.) The discriminatory power, Adjusted Rand index, and Wallace's coefficients were determined using EpiCompare version 1.0 (Ridom GmbH, Wurzburg, Germany).

RESULTS

MLVA

Amplification of eight VNTRs (SIRU01, -05, -07, -13, -15, -16, -21 (*spa*), and *sspa*) was performed on DNA of hundred clinical *S. aureus* isolates. The number of repeat units ranged from 0 to 26 (Table 1).

Table 1. Characteristics of the MLVA scheme.

Locus (size in bp)	Formula[a]	No. of RUs (% PCR negative)
SIRU 01 (55)	(*n*-157-30)/55	0 – 5 (4.5)
SIRU 05 (60)	(*n*-76-78)/60	1-22 (21.5)
SIRU 07 (56)	(*n*-27-160)/56	1 – 4 (2.2)
SIRU 13 (64)	(*n*-76-78)/64	0 – 26 (2.2)
SIRU 15 (131)	(*n*-48-174)/131	0 – 5 (0)
SIRU 21 (24)	(*n*-12-81)-16)/24	1 – 16 (0)

[a] The formula for calculating the number of repeat units (RU) per locus is as follows: (amplicon size [*n*] – size of the left flanking region – size of the right flanking region)/ size of the repeat unit.

Two loci (SIRU16, and *sspa*) were excluded from our MLVA typing scheme because no variation in the number of repeat units was observed (data not shown). The six remaining VNTR loci (SIRU01, -05, -07, -13, -15, and -21) were used for further analysis.

Seventy-three isolates yielded a complete MLVA profile, while for 27 isolates some loci were not amplified. These loci were assigned a 999 instead of a repeat unit (RU) number. The MLVA typing resulted in 76 different allelic profiles or MLVA types (MTs) (Table 2).

Table 2. MLVA allelic profiles, MTs, and *spa* types obtained from a hundred *S. aureus* with known MLST types.

		MLST	Clonal	*Spa*	MLVA	SIRU*					
No.	**Sample**	**type**	**complex**	**type**	**type**	**01**	**05**	**07**	**13**	**15**	**21**
1	S0041	7	5 [a]	91	1	0	3	3	3	3	10
2	S0033	12	12	160	2	1	7	1	3	1	7
3	S0117	717	12	160	3	1	8	1	4	1	7
4	S0061	45	45 [a]	3	4	1	2	1	4	1	8
5	S0055	22	22 [a]	223	5	2	3	1	3	1	11
6	S0134	34	30 [a]	2285 [c]	6	2	3	1	1	2	15
7	S0141	15	15 [a]	346	7	2	5	2	2	1	10
8	S0032	30	30 [a]	1945	8	2	3	2	1	2	12
9	S0296	36	30 [a]	18	9	2	3	2	1	2	11
10	S0084	149	5 [a]	2	10	2	2	1	4	1	10
11	S0168	228	228	1	10	2	2	1	4	1	10
12	S0072	228	228	1	10	2	2	1	4	1	10
13	S0077	228	228	1	10	2	2	1	4	1	10
14	S0085	228	228	1	10	2	2	1	4	1	10
15	S0088	228	228	1	10	2	2	1	4	1	10
16	S0065	228	228	1	10	2	2	1	4	1	10
17	S0071	736	5 [a]	1	10	2	2	1	4	1	10
18	S0056	228	228	1	10	2	2	1	4	1	10
19	S0108	228	228	41	11	2	2	1	4	1	14
20	S0074	228	228	41	11	2	2	1	4	1	14
21	S0087	228	228	1	12	2	2	1	2	1	10
22	S0062	225	5 [a]	3	13	2	2	2	3	2	8
23	S0063	228	228	1	14	2	2	2	4	1	10

No.	Sample	MLST type	Clonal complex	*Spa* type	MLVA type	SIRU*					
						01	05	07	13	15	21
24	S0070	228	228	1	15	2	2	2	1	0	10
25	S0031	5	5 [a]	3	16	2	3	1	3	1	8
26	S0054	34	30 [a]	369	17	2	3	1	2	2	11
27	S0118	225	5 [a]	3	18	2	3	1	4	1	8
28	S0075	225	5 [a]	3	18	2	3	1	4	1	8
29	S0079	225	5 [a]	3	18	2	3	1	4	1	8
30	S0081	225	5 [a]	3	18	2	3	1	4	1	8
31	S0022	714	30 [a]	136	19	2	3	1	2	2	13
32	S0047	715	30 [a]	166	20	2	3	1	2	2	12
33	S0050	30	30 [a]	122	21	2	3	2	1	0	8
34	S0057	225	5 [a]	3	22	2	3	2	3	2	8
35	S0053	30	30 [a]	18	23	2	3	2	2	2	11
36	S0021	713	30 [a]	18	24	2	4	2	2	2	11
37	S0043	26	25	81	25	2	5	2	10	5	8
38	S0049	239	8 [a]	275	26	2	3	1	8	1	8
39	S0112	5	5 [a]	2	27	2	3	1	4	1	10
40	S0128	5	5 [a]	2	27	2	3	1	4	1	10
41	S0042	228	228	1	27	2	3	1	4	1	10
42	S0045	247	8 [a]	52	27	2	3	1	4	1	10
43	S0132	739	101	56	28	2	4	2	6	2	9
44	S0111	247	8 [a]	2	29	2	1	1	4	1	10
45	S0113	36	30 [a]	12	30	2	3	2	1	2	10
46	S0060	45	45 [a]	15	31	3	2	2	1	1	10
47	S0059	5	5 [a]	2	32	3	2	3	1	1	10
48	S0027	8	8 [a]	9	32	3	2	3	1	1	10
49	S0029	8	8 [a]	8	32	3	2	3	1	1	10
50	S0044	8	8 [a]	8	32	3	2	3	1	1	10
51	S0083	738	8 [a]	8	32	3	2	3	1	1	10
52	S0068	8	8 [a]	8	33	3	3	2	2	2	10
53	S0028	239	8 [a]	37	34	3	2	2	26	1	7
54	S0120	684	8 [a]	37	35	3	2	2	1	1	7
55	S0178	239	8 [a]	30	36	3	2	2	0	1	6
56	S0177	239	8 [a]	30	36	3	2	2	0	1	6
57	S0058	225	5 [a]	3	37	4	9	2	1	1	8
58	S0030	15	15	84	38	4	7	2	2	5	11
59	S0107	239	8 [a]	37	39	4	2	2	1	1	7

No.	Sample	MLST type	Clonal complex	*Spa* type	MLVA type	SIRU*					
						01	05	07	13	15	21
60	S0109	8	8 [a]	8	40	4	2	3	0	1	10
61	S0025	247	8 [a]	51	41	4	22	2	1	1	11
62	S0035	247	8 [a]	51	42	4	4	2	0	1	11
63	S0034	15	15	254	43	4	7	2	4	5	8
64	S0297	8	8 [a]	8	44	4	1	3	0	1	10
65	S0298	8	8 [a]	64	45	4	2	2	0	1	10
66	S0127	239	8 [a]	138	46	4	2	2	0	1	6
67	S0121	685	8 [a]	64	47	4	2	3	1	1	10
68	S0129	247	8 [a]	75	48	4	4	2	1	1	12
69	S0069	239	8 [a]	30	49	5	2	2	1	1	6
70	S0066	735	45 [a]	305	50	5	3	2	4	2	11
71	S0051	239	8 [a]	421	51	2	3	1	4	1	6
72	S0052	45	45 [a]	693	52	2	2	1	4	1	1
73	S0039	101	101	56	53	2	2	1	4	1	9
74	S0067	254	8 [a]	9	74	999	2	2	1	1	10
75	S0037	9	singleton [b]	2176 [d]	77	4	2	999	0	1	4
76	S0300	5	5 [a]	88	80	3	10	1	999	2	11
77	S0299	72	8 [a]	126	83	3	10	999	999	2	8
78	S0295	5	5 [a]	2	85	2	999	1	4	1	10
79	S0046	228	228	1	85	2	999	1	4	1	10
80	S0076	22	22 [a]	32	87	2	999	2	4	0	16
81	S0078	22	22 [a]	32	87	2	999	2	4	0	16
82	S0089	22	22 [a]	476	89	2	999	2	2	0	11
83	S0116	97	97	527	91	2	999	3	3	1	12
84	S0024	22	22 [a]	192	93	2	999	2	7	3	13
85	S0036	45	45 [a]	1081	95	999	999	1	2	2	7
86	S0086	45	45 [a]	4	97	999	999	2	0	1	9
87	S0130	45	45 [a]	26	99	999	999	2	1	0	3
88	S0023	45	45 [a]	1933	101	999	999	2	1	1	7
89	S0166	239	8 [a]	37	103	999	999	2	0	1	7
90	S0176	239	8 [a]	37	103	999	999	2	0	1	7
91	S0167	239	8 [a]	37	105	4	999	2	0	1	7
92	S0175	239	8 [a]	37	105	4	999	2	0	1	7
93	S0026	247	8 [a]	844	107	4	999	2	0	1	8
94	S0169	247	8 [a]	51	109	4	999	2	0	1	11
95	S0110	247	8 [a]	52	111	4	999	2	0	1	10

No.	Sample	MLST type	Clonal complex	*Spa* type	MLVA type	SIRU*					
						01	05	07	13	15	21
96	S0126	572	8 [a]	51	113	4	999	2	1	1	11
97	S0119	1	1	922	115	1	999	3	1	2	6
98	S0040	188	1	189	117	1	999	3	3	5	6
99	S0115	239	8 [a]	359	119	5	999	4	3	2	9
100	S0082	737	22 [a]	5	122	2	999	2	3	0	12

*SIRU: Staphylococcal Interspersed Repeat Unit; *: values are the number of repeats (RUs) in locus; 999: no SIRU PCR amplification. [a] : Isolates belong to one of the 5 major clonal complexes (CC5, CC8, CC22, CC30 and CC45). Clonal complex is clustering of isolates based on MLST types.
[b]: Singleton: MLST types did not group into any of the clonal complexes. [c] and [d]: Two new *spa*-types observed in this study.

A minimum spanning tree showed clustering of the 76 different MLVA profiles in three dominant clusters (genogroups) of single and double locus variants C1, C2 and C3 (Figure 2). All previously assigned CC30 isolates based upon MLST belong to MLVA genogroup C1. While the genogroup C2 was dominated by CC5 and CC228 isolates, in addition to isolates that belonged to other MLST-based clonal complexes. Genogroup C3 was almost completely composed of CC8 isolates. However, some CC8 isolates fell into the two other genogroups. Isolates belonging to CC45 were scattered over the minimal spanning tree.

Spa-typing

Amplification and sequencing of protein A region of 100 human *S. aureus* yielded 50 sequence variations or *spa*-types including 2 new *spa*-types, t2285 and t2176. The number of repeats determined by MLVA for SIRU21, representing the *spa* gene, completely agreed with the number of repeats obtained by *spa*-typing for all isolates tested.

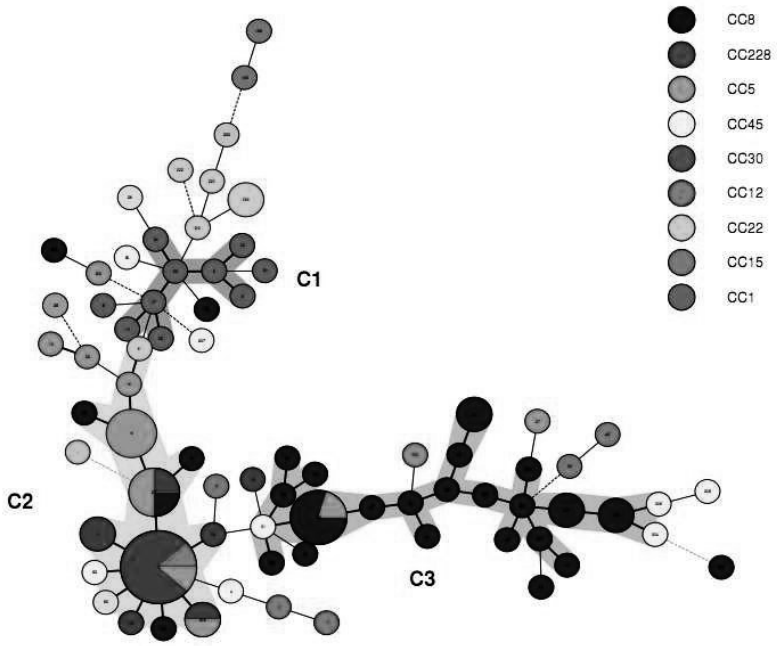

Figure 2. Population structure of *S. aureus* isolates based on MLVA. Each circle represents a different MLVA profile. Three clusters (C1, C2, and C3) were identified as shown by the three colors (blue, green, and pink). Heavy lines connecting two MTs denote a single-locus variant, thin lines denote MTs with a double-locus variant, and dotted-lines connect MTs that differ by more than two loci.

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

Discriminatory power of MLVA, MLST and *spa*-typing was determined and compared by calculating the genetic diversity (*D*) with 95% Confidence Interval (CI) of the 100 isolates typed by these three methods (Table 3). MLVA had higher level of discriminatory power compared to both MLST and *spa*-typing (0.987, 0.941 and 0.963 respectively), although the 95% CI of the genetic diversity of MLVA just overlapped with that of *spa*-typing.

Table 3. Simpson's index of diversity (*D*) and 95% confidence interval (CI).

Typing Method	Number of different type	Discriminatory index (*D*)	95% confidence interval (CI)
MLST	35	0.941	(0.922-0.96)
Spa-typing	50	0.963	(0.946-0.979)
MLVA	76	0.987	(0.977-0.997)

Total of 100 isolates tested.

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

The Adjusted Rand index for MLVA and *spa*-typing, and MLVA and MLST was 0.341 and 0.184, respectively. The Wallace's coefficients are given in Table 4 and indicated that MLVA was reasonably predictive for both MLST and *spa*-type, whereas the reverse was not true.

Table 4. Wallace's coefficients for the methods used to characterize the 100 human *S. aureus* isolates.

Typing method	MLST	*Spa*-typing	MLVA
MLST	-	0.433	0.123
Spa-typing	0.690	-	0.239
MLVA	0.554	0.677	-

Confirmation study

A confirmation study was performed to validate the MLVA scheme by comparing MLVA with PFGE, the gold standard for typing *S. aureus* isolates, and *spa*-typing, in a set of 50 *S. aureus* isolates from the Department of Infection Control and Infection Prevention of the University Medical Centre Utrecht, The Netherlands. The 50 isolates were assigned to two groups by the Department of Infection Control and Infection Prevention of the University Medical Centre Utrecht, The Netherlands, based on either phage type or PFGE type (depending on the method used by the National Institute of Public Health and the Environment, The Netherlands, at the time of initial isolation) and their time of isolation. The two groups of isolates consisted of epidemiologically related outbreak isolates and isolates with no known epidemiological link. Thirty five isolates yielded complete MLVA profiles, while 15 others showed either no amplification of some loci (999) or two PCR fragments detected for SIRU13 which was assigned 888 (isolate nr. 459 and Z548).

A total of 31 MTs, 24 *spa*-types and 30 PFGE profiles were obtained (Table 5). Simpson's indices of discriminatory power for MLVA, *spa*-typing and PFGE were 0.971, 0.908 and 0.970 respectively. This showed that also for these isolates MLVA typing had a higher discriminatory power compared to the other typing methods, although the 95% CI of MLVA overlapped with that of PFGE (0.955-0.988 for MLVA and 0.953-0.986 for PFGE). The concordance between MLVA-*spa*-typing and MLVA-PFGE was 93.5% and 97.7%, respectively.

MLVA typing distinguished 10 MTs from 22 isolates belonging to 6 outbreaks and 21 MTs in 28 unlinked isolates (Table 5). For two outbreaks (O1 and O6) MLVA, *spa*-type, and PFGE completely agreed. Four other outbreaks (O2, O3, O4, and O5) showed single locus differences in MLVA.

Outbreak O2 had an MLVA profile and *spa*-type that was shared with an unexpected isolate (593) that occurred nearly 10 years later. Outbreak O2 consisted of an isolate with a different MLVA and *spa*-type, but was originally considered an outbreak based on phage type.

Isolate nr. 529 from outbreak O3, which is a single locus variant of the other O3 outbreak isolates had a different *spa*-type, differing in only two repeats and a related but different PFGE profile. Two isolates (298 and 319) that had a similar MLVA profile and identical PFGE pattern and *spa*-type as the outbreak strain were originally not considered to be part of the outbreak based on isolation date and on phage typing. This indicates earlier circulation of related isolates preceding the outbreak that occurred a number of years later.

Isolates belonging to outbreak O4 displayed a maximum divergence of one locus difference in MLVA profile, while *spa*-type and PFGE profile were the same as for the other outbreak isolates. Another isolate (nr. 506) had the same MT and *spa*-type as the outbreak strain, but a different PFGE type and was previously considered not belonging to this outbreak, also based on its isolation date.
Isolates belonging to outbreak O5 were indistinguishable from outbreak O4 based on *spa*-type and highly related based on MLVA-profile. It should be noted that O5 isolates were taken more than 5 years later to that O4 isolates and they showed unrelated PFGE profiles.

Isolate nr. 395 showed an MLVA profile similar to the isolates of outbreak of O6 and the same phage type. PFGE pattern and *spa*-type, however, were different. Nevertheless this isolate was not considered to be part of the outbreak because of a foreign origin.

The 28 other unrelated isolates exhibited all a unique MT. In summary, MLVA was able to distinguish the different hospital outbreaks when single locus variants are considered to belong to the same outbreak and the date of isolation is taken into consideration.

Congruence calculations show that the Adjusted Rand index is 0.599 and 0.435 when MLVA is compared with PFGE and *spa*-typing for these isolates. The Wallace's coefficients show that MLVA and *spa*-typing are mutually predictive for these isolates (Table 6).

Table 5. MTs, *spa* types, and PFGE profiles of *S. aureus* isolates[a].

Isolate	Date of isolation	Phage type	PFGE cluster[c]	UMCU classification[c]	PFGE profile	*Spa* type[b]	MLVA type	No. of RUs in locus[a]					
								01	05	07	13	15	21
199	13-11-97	Z136		O1	a	2175	178	1	999	3	1	2	9
215	14-11-97	Z136		O1	a	2175	178	1	999	3	1	2	9
265	09-10-98	Z136		O1	a	2175	178	1	999	3	1	2	9
266	21-10-98	Z136		O1	a	2175	178	1	999	3	1	2	9
341	31-05-00	Z136		U	f	67	89	2	1	1	4	1	9
593	01-08-06		35	U	u	2	29	2	1	1	4	1	10
130	31-05-96	III205		O2	b	2	29	2	1	1	4	1	10
132	10-06-96	III205		O2	b	2	29	2	1	1	4	1	10
158	04-01-97	III205		O2	b	2	100	2	1	1	5	1	10
160	1997	III205		O2	b	2	236	2	1	999	4	1	10
325	11-01-00	III205		O3	b	2	29	2	1	1	4	1	10
241	23-06-98	III205		O3	e	447	53	2	2	1	4	1	9
478	01-03-04		60a	U	e	447	53	2	2	1	4	1	9
298	18-06-99	III-283		U	c	2	101	2	5	1	4	1	10
319	25-11-99	III-311		U	c	2	101	2	5	1	4	1	10
519	16-12-04		55	O4	c	2	27	2	3	1	4	1	10
524	05-01-05		55	O4	c	2	27	2	3	1	4	1	10
530	07-01-05		55	O4	c	2	27	2	3	1	4	1	10
529	05-01-05		55	O4	c1	2173	102	2	3	2	4	1	10
144	07-11-96	Z115		O5	g	8	32	3	2	3	1	1	10
145	21-02-96	Z115		O5	g	8	47	4	2	3	1	1	10
162	01-02-97	Z115		O5	g	8	32	3	2	3	1	1	10
255	15-09-98	Z115		O5	g	8	32	3	2	3	1	1	10
343	13-11-00	Z115		O5	g	8	32	3	2	3	1	1	10
506	29-06-04		18	U	h	8	32	3	2	3	1	1	10
516	06-12-04		148	O6	d	8	137	3	3	3	1	1	10
518	11-12-04		148	O6	d	8	136	3	3	3	0	1	10
520	11-12-04		148	O6	d	8	136	3	3	3	0	1	10

Isolate	Date of isolation	Phage type	PFGE cluster[a]	UMCU classification[b]	PFGE profile	*Spa* type[c]	MLVA type	No. of RUs in locus[d]					
								01	05	07	13	15	21
347	27-03-01	Z231		O7	i	52	412	4	25	2	1	1	10
363	07-04-01	Z231		O7	i	52	412	4	25	2	1	1	10
371	13-04-01	Z231		O7	i	52	412	4	25	2	1	1	10
348	27-05-01	Z231		O7	i	52	412	4	25	2	1	1	10
395	06-12-01	Z231		U	bb	1257	169	4	2	2	1	1	10
90	01-10-95	III-84		U	z	138	167	4	2	2	1	1	6
125	15-02-96	III-29		U	m	31	308	999	999	2	1	0	9
129	21-05-96	V2		U	u	1	10	2	2	1	4	1	10
284	10-11-98	III-70		U	k	24	170	4	1	3	0	1	9
331	18-02-00	III-322		U	y	2	27	2	3	1	4	1	10
393	03-12-01		NT	U	w	1932	90	2	3	2	2	2	10
401	05-02-02		147	U	l	44	275	4	999	1	3	6	14
450	09-10-03		28	U	n	728	308	999	999	2	0	1	5
459	14-11-03		NT	U	p	567	416	3	999	3	888	2	5
466	04-12-03		118a	U	t	311	88	2	13	1	4	1	9
482	16-06-04		430/426	U	x	223	235	2	999	2	3	0	11
502	23-10-04		1293	U	r	1293	179	1	999	2	2	5	8
503	28-10-04		25a	U	s	437	190	2	999	4	2	5	7
559	13-08-05		93	U	f2	729	237	2	999	2	3	2	10
561	26-10-05		218	U	aa	8	168	4	1	3	1	1	10
Z548	04-03-06		NT	U	o	11	402	3	999	3	888	2	7
570	11-03-06		99a	U	y	37	401	4	2	2	999	1	7
578	24-04-06		613	U	q	316	177	1	999	3	1	5	6
602	25-08-06		46	U	k1	8	44	4	1	3	0	1	10

[a] These classifications are compared to those previously given by the Department of Infection Control and Infection Prevention, UMCU, The Netherlands, on the basis of phage type or PFGE clustering.
[b] NT, not typeable.
[c] O1 to O6, outbreaks 1 to 6, respectively; U, unlinked epidemiological isolate.
[d] Asterisks indicate two new *spa* types observed in this study.
[e] 999, no SIRU amplification found; 888, double fragments on SIRU PCR detected.

Table 6. Wallace's coefficients for the methods used to characterize the Hygiene *Staphylococcus aureus* isolates.

Typing method	MLST	*Spa*-typing	MLVA
MLST	-	0.955	0.545
Spa-typing	0.313	-	0.276
MLVA	0.632	0.974	-

Stability of MLVA typing

To obtain insight into the stability of MLVA typing both *spa*- and MLVA-typing were performed on 14 *S. aureus* isolates recovered from 4 patients that were collected at different time points ranging from 2-7 years. Both MLVA and *spa*-typing revealed identical MT and *spa*-type for isolates from patient 3 and 4. Whereas *S. aureus* isolates recovered from patient 1 showed variation in two loci (SIRU 1 and 7) but these isolates had an identical *spa*-type. The first 3 isolates of this patient were collected over a 5 years time period, whereas the last isolate was collected 7 years after the first. Distinct genotypes as shown by distinct MT and *spa*-type were obtained from *S. aureus* isolates from patient 2. This might be explained by re-infection with a different *S. aureus* strain (Table 7). These data indicate that MLVA profiles are relatively stable over time.

Table 7. Evolution of *S.aureus* carriership based on MTs for four patients over time.

Patient	Year of isolation	Isolate	*Spa* type	MLVA type	SIRU					
					01	05	07	13	15	21
1	1996	96-121	t008	63	4	2	4	1	1	10
	1999	99-307	t008	63	4	2	4	1	1	10
	2001	01-386	t008	63	4	2	4	1	1	10
	2003	03-438	t008	31	3*	2	2*	1	1	10
2	1999	99-288	t037	35	3	2	2	1	1	7
	2001	01-346	t121	56	2*	7*	2	1	1	9*
3	1987	87-A117	t075	48	4	4	2	1	1	12
	1988	88-A208	t075	48	4	4	2	1	1	12
	1989	89-A313	t075	48	4	4	2	1	1	12
	1990	90-A358	t075	48	4	4	2	1	1	12
	1992	92-A405	t075	48	4	4	2	1	1	12
4	1996	96-129	t001	10	2	2	1	4	1	10
	1996	96-139	t001	10	2	2	1	4	1	10
	2001	01-384	t001	10	2	2	1	4	1	10

An isolate collected in 2003 from patient 1 showed two loci variations (SIRU1 and -07)

compared to the isolates collected during the previous year, even though identical *spa*-type was observed. Two isolates taken from patient 2 revealed three loci variation (SIRU01, -05 and -21) indicating these two isolates were unrelated. The different *spa*-types strengthen this finding.
The isolates are numbered according to year of isolation and strain number, e.g., 96-121 means the isolate collected in 1996 with strain number 121.
* Asterisks indicate locus variation.

DISCUSSION

The need for a rapid, less-labor intensive, accurate, reliable, reproducible, relatively cheap, and highly discriminatory typing method has increased together with the emergence and rapid spread of multiresistant bacterial pathogens worldwide. In this study, we developed and evaluated a MLVA typing scheme for human *S. aureus* and compared its discriminatory power to that of MLST and *spa*-typing. Typing of 100 European clinical isolates representing both the major clonal complexes as well as singletons showed a good typeability and excellent discriminatory power. The reliability of the methods is very good as demonstrated by the fact that the number of repeats determined by MLVA for SIRU21 completely agreed with the number of repeats obtained by *spa*-typing.

A wide variety of bacterial typing methods have been developed for determining the genetic relatedness of *S. aureus*. The method to be chosen highly depends on the questions asked (18). PFGE, although known as a gold standard, is labor intensive, technically demanding, time consuming, expensive and interlaboratory data exchange is hard to achieve. MLST is now frequently used, but there are some disadvantages such as lower discriminatory power compared to PFGE. Furthermore, MLST is relatively time consuming, expensive and this method only limited to centers that have access to the sequencing facilities (3,24,27,29). The need for sequence facilities is also a major drawback of *spa*-typing, which gained popularity last years. Recently, a number of studies have focused on the use of intergenic regions that contain repetitive DNA loci (15,28). These loci are known to be prone to generate genetic diversity by frequent acquisition and loss of tandem repeats. For epidemiological purposes, however, VNTR loci need not be too unstable, because this would mask genetic relatedness of strains and may hide/conceal an outbreak. Polymorphism of flanking regions and/or the presence of additional sequences such as IS sequences or transposable elements that

influence the result of MLVA typing should ideally be absent (15). MLVA has shown a great potential for fast and reliable typing of pathogenic bacteria especially in an outbreak situation (15). Although MLVA meets the need for rapid and reliable typing in hospital outbreaks, several drawbacks are still present. Most MLVA schemes employed a single multiplex PCR which amplified several gene targets in a single PCR reaction with a banding pattern as the experimental output, similar to that of PFGE (7,22,28). A disadvantage of this method is that it is hard to assess variation in individual repeats, including determination which locus has been amplified and calculation of the number of repeats that are present is usually not possible. This is why in this study we developed a character based MLVA scheme, which result in an allelic profile rather than a fingerprint.

A confirmation study for validation of our novel MLVA typing scheme demonstrated that MLVA was able to recognize outbreaks, that were originally based on phage typing or PFGE, by distinguishing outbreak isolates from non-outbreak isolate. It is obviously shown that some isolates could be mistaken by clustering them into an outbreak group when considered exclusively based on the phage type, whereas MLVA distinguished different MLVA types.

The Adjusted Rand index, which is measure for the congruence between methods that corrects for chance agreement, shows a somewhat lower congruence between MLVA and *spa*-typing for the European isolates than the isolates from the Hospital Hygiene used in the second part of the study. This difference is also shown by the Wallace's coefficient for MLVA compared to *spa*-typing. A high value of Wallace's coefficient indicates that partitions defined by a given method could have been predicted from the results of another method, suggesting that the methodologies are redundant. Based on this MLVA is reasonably well predictive of *spa*-type for the European isolates and highly predictive for the Hospital Hygiene isolates. This is in accordance with the higher discriminatory power for MLVA. MLVA and PFGE are only poorly predictive for each other indicating that they are not based on the same genetic signal. The relationship of MT genogroup and clonal complex as shown in Figure 2 indicates an association between MT and CC for most isolates, but notable exceptions such as isolates belonging to CC45 exist. This indicates exceptional variability in MLVA profiles in these isolates, which may either be explained by unexpected evolutionary pressure or horizontal transfer of genetic material containing one or more SIRU among ST45 isolates.

Most importantly, we have shown that MLVA was highly predictive for outbreaks and incidental isolates. However, variation of one or more loci within an outbreak isolates that had indistinguishable or closely related PFGE profile was observed. These findings are not unique to this *S. aureus* MLVA typing scheme (13,14,19-21,27). Therefore, the MLVA typing criteria used for *Enterococcus faecium* introduced by Top *et al* were followed (27). These criteria are: Indistinguishable: isolates have an identical MLVA type; Closely related: isolates have MLVA types differ only in one locus, also called single locus variant (SLV); Possibly related: isolates show MLVA types that differ in two loci, so-called double locus variants (DLV); Unrelated: isolates that have more than two loci different on their MLVA types. These criteria are similar to the Tenover criteria for PFGE and should only be applied when there is a plausible epidemiological relationship between the patients from which the isolates were obtained (25).

The MLVA profiles were stable over a 5 year period for 3 patients and only isolates obtained 7 years after the first isolate for one patient had a two loci difference. These data indicate that long term stability of the MLVA profiles is not a major concern for outbreak detection.

Summarizing, our study demonstrated that this MLVA scheme provides a fast, cheap, highly discriminatory, reproducible, stable and portable typing scheme for epidemiological tracing of both MSSA and MRSA.

Based on this finding, further study is to set up a library method similar to MLST and *spa*-typing with a MLVA Web-based database for international comparison.

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Chapter 3

Characterization of Dutch *Staphylococcus aureus* from bovine mastitis using a multiple locus variable number tandem repeat analysis

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ABSTRACT

Current typing methods for *Staphylococcus aureus* have important drawbacks. We evaluated a Multiple Locus Variable-number tandem repeat Analysis (MLVA) scheme with 6 loci which lacks most drawbacks on 85 bovine mastitis isolates from The Netherlands. For each locus the number of repeat units (RU) was calculated. Each combination of repeat units was assigned a MLVA-type (MT). We compared the MLVA typing result with Multi Locus Sequence Typing (MLST), *spa*-typing and Pulsed-Field Gel Electrophoresis (PFGE). MLVA typing resulted in 18 MTs, although 3 loci could not always be amplified. *Spa*-typing distinguished 10 *spa*-types including 3 dominant and 2 new types. PFGE showed 5 dominant profiles with 15 related profiles and 6 unique profiles. MLST showed 4 dominant STs. Some types appeared to be bovine specific. The Simpson's Indices of diversity for PFGE, MLST, *spa*-typing and MLVA were 0.887, 0.831, 0.69 and 0.781, respectively, indicating that discriminatory power of MLVA was between MLST and *spa*-typing, whereas PFGE displayed the highest discriminatory power. However, MLVA is fast and cheap when compared to the other methods. The Adjusted Rand index and Wallace's coefficient indicated that MLVA was highly predictive for *spa*-type, but not vice versa.
Analysis of the region neighboring SIRU05 showed a difference in the genetic element bordering the repeats of SIRU05 that explained the negative SIRU05 PCRs. PFGE, MLST, and MLVA are adequate typing methods for bovine-associated *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus is a major agent of contagious mastitis in diary cattle. The sources of bovine mastitis cases mostly are from bovine origin, but *S. aureus* originating from the farmer are another important source (Zadoks et al., 2002). In 2005 it was reported that each year at least 25% of all milking cows in The Netherlands suffer from clinical mastitis which is not only due to *S. aureus* (32%) as a causative agent (Lam, 2005). Many different typing methods have been used. From a "gold standard" Pulsed-Field Gel Electrophoresis (PFGE) to Multi Locus Sequence Typing (MLST) and *S. aureus*-specific staphylococcal Protein A typing known as *spa*-typing (Enright, et al., 2000; Harmsen et al., 2003; Struelens et al.,1992). However, not all methods can be used in all centers because some methods are still limited to well-equipped laboratories. Furthermore, both MLST and *spa*-typing have insufficient discriminatory power to allow accurate delineation of outbreaks and PFGE is a fingerprinting method which makes interlaboratory comparison difficult (van Belkum et al., 1995).

A limited number of molecular studies have been published that explored the population structure and genetic relationships of *S. aureus* causing bovine mastitis (de Sousa et al., 2007; Hata et al., 2006; Jørgensen et al., 2005; Katsuda et al, 2005; Reinoso et al., 2008). The results of our recent study demonstrated that a multiple locus variable number of tandem repeats (VNTR) analysis (MLVA) could be used as a fast, inexpensive, highly discriminatory, reproducible, stable and portable typing method for epidemiological tracing of human *S. aureus*. (Ikawaty et al., 2008). Therefore, we aimed to expand the use of this novel MLVA scheme to *S. aureus* isolated from clinical cases of bovine mastitis and to get insight in their genetic relationship with the human *S. aureus* population.

2. Methods

2.1. Strain collection

Eighty five *Staphylococcus aureus* isolates of clinical or subclinical cases of mastitis were included. Thirty five isolates were obtained by the Faculty of Veterinary Medicine Utrecht University, Utrecht, The Netherlands from at least 26 farms near Utrecht. Isolates from farms sampled twice were taken at least one year apart. Another 50 isolates were collected by the Animal Health Service in

Deventer, The Netherlands for the Central Veterinary Institute, Lelystad, The Netherlands from farms throughout The Netherlands. The sources were individual teat milk samples from dairy cattle from all over the country from clinical or subclinical cases of mastitis. Each isolate from CVI represents one farm, the location of the farms is unknown but they are distributed all over the Netherlands. All isolates were methicillin-susceptible *Staphylococcus aureus* (MSSA) and collected between 1988 and 2005.

2.2. Genomic DNA preparation

The isolates 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 (Macherey-Nagel) following the protocols from the manufacturer with the exception that bacterial pellet is resuspended in buffer T1. In our method, T1 buffer was replaced by freshly-made lysis buffer that contained 20mM Tris/HCl, 2mM EDTA, 1% Triton X-100, and supplemented with lysostaphin, achromopeptidase and RNase.

2.3. MLVA typing

A combination of 6 loci (SIRU01, 05, 07, 13, 15 and 21 (*spa*)) from a previous study by Hardy *et. al*. were used for MLVA typing (Hardy et al., 2006). Amplification of SIRUs (Staphylococcal Interspersed Repeat Units) was performed as described before (Ikawaty et al., 2008).

2.4. Assignment of MLVA type (MT)

The number of repeats for each locus was determined by subtracting the size of the flanking regions from the size of the amplicon followed by division by size of the repeat (Table 1). The repeat number obtained was rounded up or down to the closest integer copy number. A number string resulted from combination of repeat units from SIRU01, 05, 07, 13, 15 and 21 was obtained after calculating the number of repat units of all loci. This was considered an allelic profile and used for the assignment of an MLVA type (MT).

2.5. Spa-typing

Amplification and sequencing of the repeat region of *S. aureus* Protein A gene (*spa*) was performed by using a specific primer set as described (Harmsen et al., 2003). The amplicon was sequenced using BigDye terminator version 3.1 on ABI 3100 sequencer (Applied Biosystem). BioNumerics (version 3.5; Applied Maths) was used to analyze the obtained sequences and to assign the *spa*-types. Novel *spa*-types were submitted to the Ridom SpaServer database (www.SpaServer.ridom.de).

2.6. Pulsed Field Gel Electrophoresis

Bacterial isolates were genotyped by PFGE as described previously (Tenover et al., 1995). Digestion of chromosomal DNA was performed overnight using the restriction enzyme *Sma*I at a temperature 25^{o}C. Fragments were separated on 1% gel. Isolate relatedness was determined using the Tenover criteria (Tenover et al., 1995).

2.7. MLST analysis

Determination of the sequence type of 85 *S. aureus* from bovine mastitis were performed as described (Enright, et al., 2000) and data were analyzed using BioNumerics software and the MLST database (www.mlst.net). The outcome of MLST of bovine mastitis *S. aureus* was compared with the total population of human *S. aureus* isolates from our database (data not shown) and the MLST.net database.

2.8. Comparison of MLVA, spa-typing, MLST and PFGE

BioNumerics software was used as a tool for clustering the observed MLVA types (MTs) and MLST types (STs). The discriminatory power of the typing methods was calculated by using EpiCompare version 1.0 (Ridom GmbH, Wurzburg, Germany) as well as for the determination of Adjusted Rand index and Wallace's coefficients.

2.9. Analysis of SIRU05 locus

Further analysis of the SIRU05 locus was performed by PCR and sequencing using specific primer sets (lysR- For: 5'-GGA AGC AGA TTT AGG TTA TG-3' and fosB-lys-Rev: 5'-CCA GTC AAT AGC AAT TTT CC-3' for amplification fragment A; lysR-For: 5'-TTT GTT CAT CTT GGC TTA GG-3' and ISRX-lys-Rev: 5'-GGA AGT TAC AAT CAT TTG CG-3' for fragment B) based on reference strain of bovine *S. aureus* RF122 (Figure 1). DNA sequencing was performed as described for *spa*-typing.

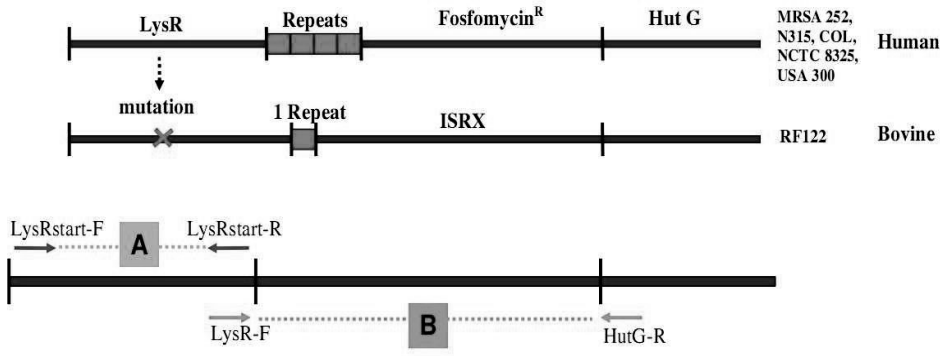

Figure 1. Strategy for analyzing the region bordering the SIRU5 locus. The bovine *S. aureus* (BSA) RF122 reference strain showed a different genetic structure compared to human *S. aureus* reference strains. The genetic element at the right of the repeat region consists of a fosfomycin resistance gene in human *S. aureus* and was replaced by insertion site region X (IS*RX*) in RF122. A single nucleotide mutation in *lysR* gene region was present in RF122. The primers were designed to detect the presence/absence of a single nucleotide mutation (fragment A) by DNA sequencing and the presence of fosfomycin resistance gene or IS*RX* structure (fragment B).

3. Results

3.1. MLVA

Among 6 SIRUs used in this typing method, no amplifications were detected for SIRU13, 07 and 05 in 47.6%, 91.8% and 100% of the cases, respectively (Table 1). Variations in the number of repeat units ranged from 0 to 12 repeat units (Table 1). Absence of PCR amplification was considered as giving a null allele and is assigned 999 for the repeat number (Table 2). The MLVA typing of 85

isolates produced 18 different allelic profiles or MLVA types . MT102 was most common with 35 isolates, followed by MT112 (n=17), and MT118 (n=8).

Table 1. Size of the MLVA loci, formula for calculating the number of repeat units (RU) per locus, typeability of MLVA and variation in repeat units observed.

Locus (size in bp)	Formula	Number (%) PCR negative	No. of repeats (RU)*
SIRU01 (55)	(n-157-30)/55	0 (0.0)	1 – 6
SIRU05 (60)	(n-76-78)/60	85 (100)	-
SIRU07 (56)	(n-27-160)/56	78 (91.8)	2 – 3
SIRU13 (64)	(n-76-78)/64	49 (47.6)	1 – 5
SIRU15 (131)	(n-48-174)/131	0 (0.0)	0 – 3
SIRU21 (24)	(n-12-81)-16)/24	0 (0.0)	2 – 12

n: Size of fragment in bp; * : values are in repeat unit (RU)

3.2. Spa-typing

Ten *spa*-types were obtained from 85 isolates, including 2 new *spa*-types (t2112 and t2248). Seventy three isolates belonged to three dominant *spa*-types, t529 (n=39), t543 (n=22) and t524 (n=17) (Table 2). The number of repeats obtained by *spa*-typing for all isolates tested were corresponding with the number of repeats determined by MLVA for SIRU21.

3.3. Pulsed-Field Gel Electrophoresis

DNA of 85 isolates was digested with *Sma*I and showed 26 PFGE profiles, 5 groups of closely related PFGE profiles (5 dominant profiles with 15 related to dominant profiles) and 6 unique PFGE profiles (Figure 2).

3.4. MLST

Sequencing of the seven housekeeping genes of all isolates identified 4 dominant MLST types (STs), ST 504 (n=24), ST 479 (n=21), ST71 (n=11), ST 151 (n=11), and 11 STs that have not been described previously in the database at http://saureus.mlst.net (Table 2). A minimum spanning tree of the ST of the 85 isolates compared to the whole population of *S. aureus* showed 3 distinct clusters with bovine mastitis *S. aureus* isolates although some isolates clustered with known human sequence types (Figure 3).

Table 2. Comparison of PFGE, MLST, *spa*-typing and MLVA results.

No	Isolate	Date of isolation	Source	PFGE profile	MLST type	*Spa*-type	MLVA type	SIRU[a]					
								01	05	07	13	15	21
1	S0416	04/2004	FVM	E	504	t529	102	1	999	999	999	0	2
2	S0409	05/2005	FVM	E	151	t529	102	1	999	999	999	0	2
3	S0333	30/09/2005	CVI	D	504	t529	102	1	999	999	999	0	2
4	S0334	17/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
5	S0335	17/10/2005	CVI	E	151	t529	102	1	999	999	999	0	2
6	S0338	03/10/2005	CVI	E	151	t529	102	1	999	999	999	0	2
7	S0341	28/09/2005	CVI	D1	504	t529	102	1	999	999	999	0	2
8	S0343	28/09/2005	CVI	E	151	t529	102	1	999	999	999	0	2
9	S0347	10/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
10	S0350	03/10/2005	CVI	D2	151	t529	102	1	999	999	999	0	2
11	S0351	17/10/2005	CVI	D1	151	t529	102	1	999	999	999	0	2
12	S0352	14/10/2005	CVI	D2	151	t529	102	1	999	999	999	0	2
13	S0353	13/10/2005	CVI	D1	151	t529	102	1	999	999	999	0	2
14	S0357	03/10/2005	CVI	E	504	t529	102	1	999	999	999	0	2
15	S0358	28/09/2005	CVI	E	151	t529	102	1	999	999	999	0	2
16	S0364	30/09/2005	CVI	E	504	t529	102	1	999	999	999	0	2
17	S0365	30/09/2005	CVI	D1	504	t529	102	1	999	999	999	0	2
18	S0367	03/10/2005	CVI	D2	1122[b]	t529	102	1	999	999	999	0	2
19	S0368	03/10/2005	CVI	E	504	t529	102	1	999	999	999	0	2
20	S0370	28/09/2005	CVI	D1	1123[b]	t529	102	1	999	999	999	0	2
21	S0371	17/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
22	S0374	17/10/2005	CVI	D2	151	t529	102	1	999	999	999	0	2
23	S0375	17/10/2005	CVI	D3	504	t529	102	1	999	999	999	0	2
24	S0376	25/10/2005	CVI	E	504	t529	102	1	999	999	999	0	2
25	S0377	25/10/2005	CVI	D1	504	t529	102	1	999	999	999	0	2
26	S0378	25/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
27	S0379	25/10/2005	CVI	D	504	t529	102	1	999	999	999	0	2
28	S0381	25/10/2005	CVI	D	1124[b]	t529	102	1	999	999	999	0	2
29	S0389	03/1995	FVM	D	504	t529	102	1	999	999	999	0	2
30	S0390	03/1997	FVM	D	1120[b]	t529	102	1	999	999	999	0	2
31	S0396	03/2002	FVM	D1	504	t529	102	1	999	999	999	0	2
32	S0417	04/2004	FVM	D2	504	t529	102	1	999	999	999	0	2
33	S0424	01/2006	FVM	D	504	t529	102	1	999	999	999	0	2
34	S0425	01/2006	FVM	E	504	t529	102	1	999	999	999	0	2
35	S0435	1988	FVM	D	504	t529	102	1	999	999	999	0	2
36	S0340	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
37	S0342	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3

No	Isolate	Date of isolation	Source	PFGE profile	MLST type	*Spa*-type	MLVA type	SIRU[a]					
								01	05	07	13	15	21
38	S0344	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3
39	S0345	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
40	S0346	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
41	S0348	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
42	S0349	10/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
43	S0354	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
44	S0355	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
45	S0356	03/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
46	S0360	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3
47	S0361	28/09/2005	CVI	A	479	t543	112	2	999	999	2	0	3
48	S0369	03/10/2005	CVI	A	1118[b]	t543	112	2	999	999	2	0	3
49	S0372	17/10/2005	CVI	A	479	t543	112	2	999	999	2	0	3
50	S0373	17/10/2005	CVI	A	1118[b]	t543	112	2	999	999	2	0	3
51	S0414	09/2003	FVM	A	479	t543	112	2	999	999	2	0	3
52	S0422	01/2005	FVM	A	479	t543	112	2	999	999	2	0	3
53	S0398	10/1998	FVM	A	479	t543	114	2	999	999	1	0	3
54	S0332	12/10/2005	CVI	A1	479	t543	114	2	999	999	1	0	3
55	S0339	03/10/2005	CVI	A	479	t543	114	2	999	999	1	0	3
56	S0391	04/2002	FVM	A	479	t543	114	2	999	999	1	0	3
57	S0388	1994	FVM	F3	1119[b]	t524	118	4	999	999	3	1	2
58	S0401	01/1999	FVM	B3	1125[b]	t524	118	4	999	999	3	1	2
59	S0427	1990	FVM	B	1129[b]	t524	118	4	999	999	3	1	2
60	S0428	1989	FVM	F	71	t524	118	4	999	999	3	1	2
61	S0429	1989	FVM	G	71	t524	118	4	999	999	3	1	2
62	S0430	1989	FVM	F1	71	t524	118	4	999	999	3	1	2
63	S0432	1989	FVM	B	71	t524	118	4	999	999	3	1	2
64	S0412	06/2003	FVM	B	71	t524	118	4	999	999	3	1	2
65	S0362	30/09/2005	CVI	B6	71	t524	125	5	999	999	3	1	2
66	S0433	1989	FVM	B1	71	t524	125	5	999	999	3	1	2
67	S0434	1989	FVM	B	71	t524	125	5	999	999	3	1	2
68	S0411	06/2003	FVM	B2	1127[b]	t524	125	5	999	999	3	1	2
69	S0413	07/2003	FVM	B5	1128[b]	t524	125	5	999	999	3	1	2
70	S0420	10/2004	FVM	D1	504	t529	125	5	999	999	3	1	2
71	S0418	08/2005	FVM	E1	504	t529	104	1	999	999	3	1	2
72	S0410	05/2003	FVM	B4	1126[b]	t524	104	1	999	999	3	1	2
73	S0419	11/2004	FVM	C	71	t524	79	6	999	999	3	1	2
74	S0421	11/2004	FVM	D1	151	t529	79	6	999	999	3	1	2
75	S0363	30/09/2005	CVI	F4	71	t524	76	5	999	999	3	0	2

No	Isolate	Date of isolation	Source	PFGE profile	MLST type	*Spa*-type	MLVA type	SIRU[a]					
								01	05	07	13	15	21
76	S0436	1990	FVM	H	97	t2174	82	6	999	2	5	2	6
77	S0387	1994	FVM	D1	504	t529	106	1	999	3	999	0	2
78	S0426	1990	FVM	B2	97	t1236	108	1	999	2	5	3	10
79	S0366	03/10/2005	CVI	I	1121[b]	t127	110	1	999	3	2	2	7
80	S0392	04/2002	FVM	A	479	t2248	116	2	999	999	1	0	6
81	S0337	13/10/2005	CVI	B2	97	t2112	120	4	999	2	5	2	11
82	S0431	1989	FVM	F2	97	t521	121	4	999	3	3	2	12
83	S0336	17/10/2005	CVI	J	71	t524	123	4	999	999	2	1	2
84	S0423	11/2005	FVM	A	479	t543	127	5	999	999	3	1	3
85	S0393	08/2001	FVM	K	124	t224	129	5	999	3	5	1	8

CVI: Central Veterinary Institute, Lelystad, The Netherlands.
FVM: Faculty of Veterinary Medicine Utrecht University, Utrecht, The Netherlands.
[a]: values are in repeat units (RU); [b]: new MLST type; 999: no amplification of SIRU.

3.5. Comparison of MLVA, spa-typing, PFGE and MLST

Discriminatory power of the four typing methods, PFGE, MLST, *spa*-typing and MLVA was determined by calculating Simpson's index of diversity with 95% Confidence Interval (CI) of the isolates typed by these methods. PFGE showed higher discriminatory power compared to MLST, *spa*-typing, and MLVA (0.887, 0.831, 0.69 and 0.781, respectively) (Table 3), but the 95% CI of MLVA overlapped with those of *spa*-typing and MLST. It is remarkable that all 3 major *spa*-types contained multiple ST (Table 2).

Table 3. Simpson's index of diversity and 95% confidence interval.

Typing Method	Number of different types	Discriminatory index	95% confidence interval
PFGE	26	0.887	0.847 - 0.926
MLST	18	0.831	0.787 - 0.875
Spa-typing	10	0.69	0.627 - 0.752
MLVA	18	0.781	0.71 - 0.852

Total of 85 isolates tested.

3.6. Analysis of the neighboring region of SIRU05

SIRU05 could not be amplified for any of the isolates. Therefore, the presence of sequences bordering SIRU05 was investigated. In human-derived SIRU05-positive strains SIRU05 is flanked by a LysR regulatory protein family gene at one side and a fosfomycin resistance encoding gene (*fosB*) followed by the *hutG* gene

which putatively encodes a formimidoylglutamase. In the sequenced bovine strain RF122 the fosfomycin resistance gene is replaced by insertion sequence IS*RX*. One primer set was used to amplify the *lysR* region (fragment A) and a second set was used to amplify the region between the *lysR* gene and *hutG* (Figure 1). All isolates were positive for amplification of fragment A and B. Sequencing of fragment A and B of 10 isolates showed a single nucleotide mutation in the *lysR* gene resulting a premature stop-codon and the absence of IS*RX*. A new primer designed in the *hutG* gene which was combined with the original primer of SIRU05 in the *lysR* gene did not show differences in the number of repeat units for this locus.

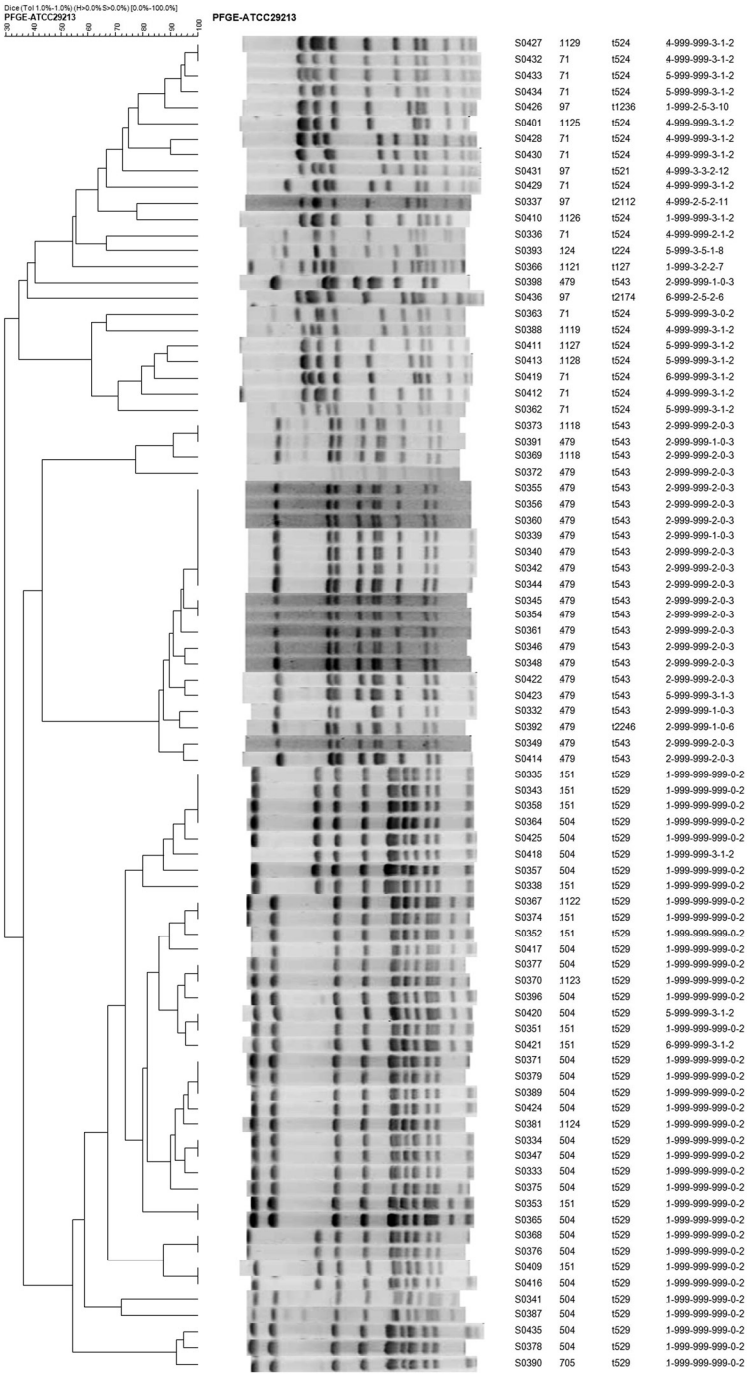

Figure 2. Dendrogram containing PFGE patterns of 85 MSSA strains collected from bovine. At the 50% similarity level, seven branches are distinguished. ID: isolate ID; ST: MLST type.

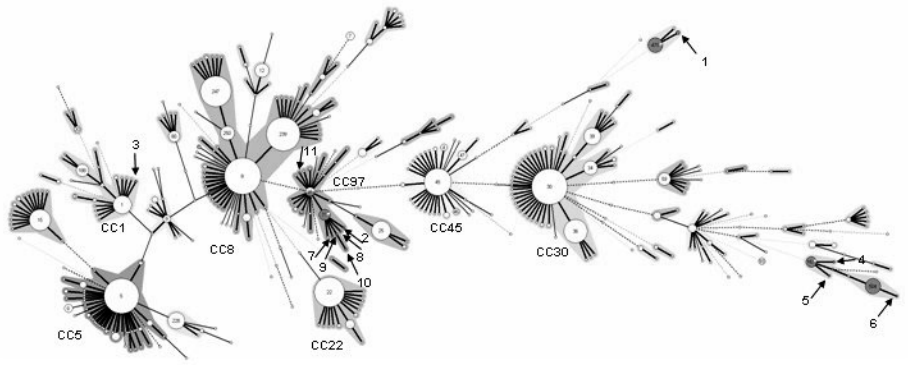

Figure 3. Minimum spanning tree of *S. aureus* based on MLST. Five major clonal complexes are present within the *S. aureus* population: CC5, CC8, CC22, CC30 and CC45. Each circle represents a different MLST type. Single locus variant STs are connected by a thick line, double locus variant STs by a thin line, triple locus variant STs by a dark grey dashed line, and STs with more than 3 loci variant are connected by a light grey dashed line. Red circles indicate *S. aureus* isolated from bovine mastitis. Arrows indicated new MLST types observed in this study: 1: ST 1118; 2: ST 1119; 3: ST 1121; 4: ST 1122; 5: ST 1123; 6: ST 1124; 7: ST 1125; 8: ST 1126; 9: ST 1127; 10: ST 1128; 11: ST 1129.

3.7. The congruence between PFGE, MLST, spa-typing and MLVA

Adjusted Rand's and Wallace's coefficients were calculated to explore the concordance between typing methods (Table 4 and 5). The Adjusted Rand's coefficient for the comparison of the clustering by MLVA and PFGE, MLVA and MLST, and MLVA and *spa*-typing was 0.385, 0.442 and 0.758, respectively. Considering MLVA as the test typing method for comparison, the value of Wallace's coefficients showed that MLVA could only poorly predict the PFGE and MLST type. The probability of two strains having the same MLVA type and sharing the same *spa*-type was 99% (Wallace's coefficient 0.991), whereas the reverse was reasonably predictive (Wallace's coefficient 0.699). This finding reflects that MLVA was less discriminatory than MLST and PFGE.

We observed variation of *spa*-type and MT within the same MLST type as shown by ST 97 and 479 isolates, although an identical *spa*-type isolates also showed variation of STs that were closely related (single locus variants or SLVs) to possibly related (3 loci different).

Identical t524 isolates had new STs: ST1119, 1125, 1126, 1127, and 1129 that were SLVs and ST1128 that was a double locus variant (DLV) of ST71.

Table 4. Adjusted Rand's coefficients for the methods used to characterize the 85 bovine mastitis *Staphylococcus aureus* isolates.

Typing method	PFGE	MLST	*Spa*-typing	MLVA
PFGE	-			
MLST	0.479	-		
Spa-typing	0.405	0.583	-	
MLVA	0.385	0.442	0.758	-

Table 5. Wallace's coefficients for the methods used to characterize 85 bovine mastitis *Staphylococcus aureus* isolates.

Typing method	PFGE	MLST	*Spa*-typing	MLVA
PFGE	-	0.684	0.941	0.699
MLST	0.459	-	0.957	0.63
Spa-typing	0.344	0.521	-	0.699
MLVA	0.362	0.486	0.991	-

4. Discussion

MLVA has shown great potential for fast and reliable typing of pathogenic bacteria. Our previous study demonstrated that a newly developed MLVA scheme for human *S. aureus* had higher discriminatory power compared to PFGE, MLST and *spa*-typing (Ikawaty et al., 2008). A major advantage of the proposed MLVA scheme is that it requires only simple laboratory equipment and is fast and relatively cheap to perform. This scheme provides better and more timely access to typing of bovine mastitis. This allows more adequate surveillance of mastitis and the identification of particular virulent or epidemic strains. Early recognition of these strains may help to initiate more timely therapy and other interventions to prevent further spread. In this study, we extended the use of the MLVA typing scheme for human *S. aureus* to bovine *S. aureus* from clinical mastitis. Eighty five isolates represented regional (n=35) and national (n=50) *S. aureus* isolates. The isolates were not considered to belong to local outbreaks as 90% of the isolates were obtained on different farms whereas the remaining isolates were obtained at least one year apart when sampled on the same farm. The scheme showed good typeability although 3 loci (SIRU05, 7 and 13) were not always amplified. SIRU21 had the most variance in the number of repeat units, which is important in typing by MLVA.

We observed no particular difference between regionally and nationally obtained *S. aureus* bovine mastitis strains in terms of PFGE profile, MLST and *spa*-types,

except for one MLVA type (MT 118) that was only present among regionally obtained isolates, but due to the limited number of isolates further analysis using more samples is needed. The number of repeat units obtained by amplification of SIRU21, having the smallest repeat unit, agreed completely with the number of repeats obtained by DNA sequencing for *spa*-typing indicating the reliability of the determination of the number of repeat units.

SIRU05 was analyzed more in depth, since it could not be amplified in any of the bovine isolates including 4 isolates with ST97 that were already known as possibly human derived (Smith et al., 2005a; Sung et al., 2008). PCR of the region neighboring SIRU05 demonstrated that all isolates were fragment A and B positive. Sequencing of the fragments of 10 isolates showed that IS*RX* and the fosfomycin resistance element were lacking. The difference in the genetic element bordering the repeats of SIRU05 explains the negative result of SIRU05 PCR, since the primer chosen in the fosfomycin resistance element could not anneal to the IS*RX* element or the *hutG* gene. Amplification of SIRU05 with a primer chosen in *hutG* showed no variation in the number of repeat units. SIRU05 does not contribute to the discrimination of the isolates tested and can be omitted from the scheme for bovine mastitis isolates. It should be noted that the primer sets used in this MLVA scheme were developed based on human *S. aureus*. LysR is a member of the largest family of bacterial activator/regulator proteins (Henikoff et al.,1988; Zaim and Kierzek. 2003). The LysR protein contains a substrate recognition site and a helix-turn-helix DNA-binding motif crucial for protein-DNA interaction (Zaim and Kierzek. 2003). In bovine *S. aureus* a stop-codon is present, which prevents transcription of the substrate domain. This should inactivate LysR leading to different expression patterns in bovine strains compared to human strains.

A minimum spanning tree based on MLST showed that clustering of bovine *S. aureus* was different than for isolates from the human population. The difference between human and bovine derived isolates has been suggested to be caused by tissue specificity by some authors (Gilbert et al., 2006; Smith et al., 2005a) while most authors assumed host specificity among *S. aureus* clones (de Sousa et al., 2007; Kapur et al.,1995). Two of the three dominant MLST types (ST151 and 504) were clustered together while ST479 was distantly related to ST151 and ST504. They were not related to human–derived *S. aureus*. Eleven isolates from both regional and national origin were ST71 which is known to be a bovine-associated strain from The Netherlands (Smith et al., 2005a). Interestingly, the 11

new STs isolates fell into a cluster which was not related to the known bovine STs. Little sharing of strains between the bovine and human population has been reported (Kapur et al.,1995). A similar finding was made by Rabello et al for Brazilian isolates (Rabello et al., 2007).

The ST or clonal complexes (CC) of bovine mastitis isolates from The Netherlands differed from those described elsewhere with the exception of 4 isolates (S0337, S0426, S0431 and S0436) that belonged to CC97. CC97 isolates have previously been reported to be obtained from humans (Feil et al., 2003). In Brazil CC97 and CC127 were predominant (Rabello et al., 2007). CC97 was also present in isolates from the UK, the USA and Chile (Smith et al., 2005a; Smith et al., 2005b). A different set of STs was obtained from milk in Norway where ST130 (CC3), ST133 and ST132 (CC1) were predominant (Jørgensen et al., 2005).

The highest discriminatory power was obtained by PFGE followed by MLST, MLVA and *spa*-typing. Because SIRU05 yielded no usable data a lower discriminatory power of MLVA compared to the *spa*-typing was expected. But the results demonstrated that MLVA had a higher Simpson's index of diversity than *spa*-typing.

We observed variations in related STs within the same *spa*-type as shown in Table 2. The limited number of *spa*-types might suggest selective pressure on the *spa* gene and it may explain their maintenance in the population of bovine-derived *S. aureus*. This finding suggests that *spa*-typing is not a useful method to compare bovine mastitis isolates.

Transfer of antibiotic resistance from human to animal isolates or the other way around has been a major concern (Pesavento et al.,2007; Zadoks et al., 2002). Host specificity of clones may reduce the chance that human-derived antibiotic-resistant *S. aureus* isolates are transmitted to cattle, although bovine mastitis may occasionally be caused by human-derived isolates. Typing of *S. aureus* from bovine mastitis will be required to monitor potentially changing population dynamics. Based on our data PFGE, MLST, and MLVA are adequate typing methods for international studies and local studies of bovine *S. aureus* isolates involved in mastitis. However, PFGE and MLST are more time-consuming and/or expensive than MLVA.

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Chapter 4

The application of a multiple locus variable number of tandem repeat (VNTR) analysis (MLVA) typing scheme to pig-related methicillin-resistant *Staphylococcus aureus* (ST398)

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ABSTRACT

We evaluated a Multiple Locus Variable-number tandem repeat Analysis (MLVA) typing scheme for pig-related *Staphylococcus aureus.* PCR was performed on 6 loci called Staphylococcal Interspersed Repeat Unit (SIRU) for fifty pig-related MRSA ST398 isolates collected from the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. All isolates showed a product in the MLVA PCR except for SIRU5 and interestingly, we observed a double fragment for SIRU13 PCR product for all isolates. The MLVA revealed 6 MLVA types with variation in SIRUs ranging from 2 – 7 repeat units. Five *spa*-types were observed including 1 new type. The number of repeat units obtained by *spa*-typing agreed with SIRU21 PCR. Simpson's index diversity of MLVA and *spa*-typing showed that both methods were equal in terms of discriminatory power, 0.842 and 0.74, respectively. Adjusted Rand coefficient and Wallace's coefficient indicated that both methods were predictive for each other. MLVA and *spa*-typing can be used to type pig-related *S. aureus* isolates. The double fragment obtained on SIRU13 PCR can be used as a specific marker for quick detection of pig-related MRSA.
Sequence analysis of the double fragment obtained from SIRU13 showed a highly complex and variable repeat structure. We speculate that SIRU13 sequences are possibly involved in regulation of methicillin resistance.

INTRODUCTION

Sequence type (ST) 398 is regarded as emerging. Studies in The Netherlands showed that more than 20% of the pig farmers and 39% of slaughterhouse pigs carried methicillin-resistant *Staphylococcus aureus* (MRSA) belonging to ST398 (4,25). The emergence of ST398 MRSA was not restricted to The Netherlands, but has also been described elsewhere such as other countries in Europe, North America, and Asia (5,12,14,18,19). Although transmission is primarily between animals, contact with livestock, especially pigs, has been identified as a risk factor for MRSA ST398 carriage (2,10,17,18,20,22).

As for human *S. aureus*, pig-related MRSA has been characterized by several molecular typing methods including Amplified Fragment Length Polymorphism (AFLP) analysis, Multilocus Sequence Typing (MLST), *S. aureus*-specific staphylococcal protein A (*spa*) typing, and Pulsed Field Gel Electrophoresis (PFGE) (2,16,18). However, pig-related methicillin-resistant ST398 were non-typeable by PFGE using *Sma*I due to the presence of 5-methylcytosine in its recognition sequence CCCGGG which prevents DNA digestion (3). AFLP showed that ST398 isolates form a distinct cluster compared to the overall population of *S. aureus*, but AFLP could not distinguish isolates from pigs and pig farmers (15). Different *spa*-types, including t011, t034, t108, t567, t899 and t939 have been described for ST398 isolates (3,13,18).

Our previous study demonstrated the value of a multiple locus variable number of tandem repeats (VNTR) analysis (MLVA) as a fast, inexpensive, highly discriminatory, reproducible, stable, and portable typing method for the epidemiological tracing of human *S. aureus* (9). The method was also applicable for bovine mastitis *S. aureus* (8,9). In this study, we evaluated the use of the MLVA scheme to pig-related methicillin-resistant *S. aureus* isolates. Results showed a double amplification fragment for SIRU13 and we analysed the genomic region of SIRU13. The gene down-stream of SIRU13 was identified by Kondo et al to induce Eagle-type methicillin resistance when cloned into *S. aureus* N315, an MRSA with low *mecA* expression due to regulation by an intact *mecI* regulator locus. It is possibly also involved in the conversion of heterogeneous to homogenous expression of methicillin resistance (11).

MATERIALS AND METHODS

Bacterial strains

Fifty pig-related methicillin-resistant *S. aureus* isolates were obtained by The Faculty of Veterinary Medicine, Utrecht University, The Netherlands. Ten isolates were obtained from farmers and 40 were from pigs, taken from 8 different farms located in three provinces, Utrecht, Noord-Brabant, and Gelderland in the period of October and November 2006. These isolates had an identical MLST type, ST398.

DNA preparation

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 (Macherey-Nagel) following the protocol from the manufacturer.

MLVA typing and assignment of MLVA type (MT)

A combination of 6 loci (SIRU01, 05, 07, 13, 15, and 21 (*spa*)) from a previous study by Hardy *et al*. were used for MLVA typing (6). Amplification of SIRUs (Staphylococcal Interspersed Repeat Units) and assignment of MLVA type were performed as previously described (9).

Spa-typing

Amplification and sequencing of the repeat region of the *S. aureus* protein A gene (*spa*) was performed by using a specific primer set as described by Harmsen *et al*. (7). The amplicon was sequenced using BigDye terminator version 3.1 on an ABI 3100 sequencer (Applied Biosystems). BioNumerics (version 3.5; Applied Maths) was used to analyze the obtained sequences and to assign the *spa*-types. Novel *spa*-types were submitted to the Ridom SpaServer database (www.SpaServer.ridom.de).

Comparison of MLVA and *spa*-typing

Clustering the observed MLVA types and *spa*-types was performed using BioNumerics software. Further comparative analysis of both typing methods by calculating the Simpson's index of diversity, the Adjusted Rand index and Wallace's coefficients was performed with EpiCompare version 1.0 (Ridom GmbH, Wurzburg, Germany).

Structural analysis of the double amplification products of SIRU13

The double amplification products of SIRU13 (Fig.1) were sequenced. The SIRU13 regions of strains S0385 and CV55 were partly sequenced using BigDye terminator version 3.1 on an ABI 3100 sequencer and partly by data obtained using 454 pyrophosphate sequencing technology by Roche Applied Sciences (Roche Diagnostics, Penzberg, Germany). Subsequently, resulting 454 reads were assembled in contigs using the 454 Newbler assembler. S0385 is a pig-related human clinical methicillin-resistant *S. aureus* isolate that caused endocarditis and belongs to ST398. CV55 is a human clinical methicillin-susceptible *S. aureus* ST398 isolate from the Cape Verde Islands (1).

The data were compared with sequence data from completed *S. aureus* genomes, including the published genomes MW2, MSSA476, Mu3, Mu 50, USA300 from Houston and San Francisco, JH-1 and JH-9, COL, MRSA252, N315, NCTC8325, Newman, and the bovine strain RF122 and the unpublished sequences of WKZ-1 and WKZ-2. The latter two isolates are human MSSA and MRSA isolates, respectively, that belong to ST30 (21).

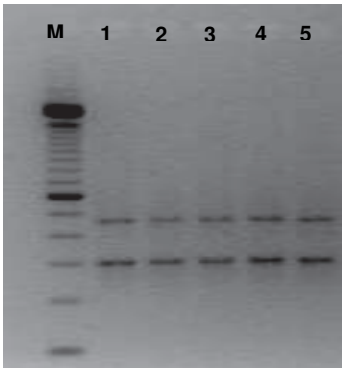

Figure 1. PCR amplification products of SIRU13 using ST398 isolate DNA.
M: 100 bp marker
1-5: pigs-associated MRSA isolates

RESULTS

MLVA.

Amplification of the SIRUs yielded PCR products for all these loci except for SIRU05. MLVA typing of 50 isolates resulted in 6 MTs. The six MLVA genogroups differed in at most 2 loci (Table 1). The variation in the number of repeat units (RU) ranged from 2 to 7. Interestingly, all isolates showed a double amplification product of SIRU13. The size difference between amplification products was the same for all isolates; therefore the number of RUs of SIRU13 was determined based on the smallest fragment. Table 1 shows that isolates from farmers share identical *spa*- and MLVA types with the pigs from the same provinces. In addition, isolates from 2 different provinces could harbor the same *spa*- and MLVA types.

Spa-typing.

Spa-typing yielded 5 *spa*-types including 1 new *spa*-type. The number of repeat units obtained by *spa*-typing agreed with the number of SIRU21 repeats calculated from the PCR (Table 1).

Comparison of MLVA and *spa*-typing.

The discriminatory power of both MLVA and *spa*-typing was determined by calculating Simpson's index of diversity with 95% confidence interval (CI) of the isolates typed by these methods. We observed that the discriminatory power of MLVA and *spa*-typing were 0.842 (CI 0.8-0.883) and 0.74 (CI 0.684-0.795), respectively.

Concordance between MLVA and *spa*-typing.

An identical Adjusted Rand index (0.697) for MLVA and *spa*-typing was observed. Wallace's coefficients were 1.00 and 0.608 for both MLVA and *spa*-typing, respectively. This result indicates that MLVA predicts the *spa*-type very well (100%), while the vice versa result indicates that two isolates with the same *spa*-type will share the same MT in 61% of the cases. This finding indicates MLVA more discriminatory than *spa*-typing.

Table 1. *Spa*-types, MLVA types and MLVA allelic profiles obtained from fifty pigs associated methicillin-resistant *S. aureus* ST 398 collection of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

No.	Sample	Source	Prov.[a]	*Spa*-type	MLVA type	SIRU[b]					
						01	05	07	13[c]	15	21
1	S0623	Pig	NB	t567	413	3	999	3	3	2	5
2	S0624	Pig	NB	t567	413	3	999	3	3	2	5
3	S0625	Pig	NB	t567	413	3	999	3	3	2	5
4	S0626	Pig	NB	t567	413	3	999	3	3	2	5
5	S0627	Pig	NB	t567	413	3	999	3	3	2	5
6	S0628	Pig	NB	t567	413	3	999	3	3	2	5
7	S0629	Pig	NB	t567	413	3	999	3	3	2	5
8	S0630	Pig	NB	t567	413	3	999	3	2	2	5
9	S0631	Pig	NB	t567	413	3	999	3	2	2	5
10	S0632	Farmer	NB	t567	413	3	999	3	2	2	5
11	S0633	Farmer	NB	t567	413	3	999	3	2	2	5
12	S0696	Pig	NB	t567	413	3	999	3	3	2	5
13	S0697	Pig	NB	t567	413	3	999	3	3	2	5
14	S0698	Pig	NB	t567	413	3	999	3	3	2	5
15	S0699	Pig	NB	t567	413	3	999	3	3	2	5
16	S0700	Farmer	NB	t567	413	3	999	3	3	2	5
17	S0701	Farmer	NB	t567	413	3	999	3	3	2	5
18	S0702	Farmer	NB	t567	413	3	999	3	2	2	5
19	S0703	Farmer	NB	t567	413	3	999	3	2	2	5
20	S0635	Pig	NB	t108	414	3	999	3	2	2	6
21	S0636	Pig	NB	t108	414	3	999	3	2	2	6
22	S0637	Pig	NB	t108	414	3	999	3	2	2	6
23	S0638	Pig	NB	t108	414	3	999	3	3	2	6
24	S0639	Pig	NB	t108	414	3	999	3	3	2	6
25	S0640	Pig	NB	t108	414	3	999	3	3	2	6
26	S0641	Pig	NB	t108	414	3	999	3	3	2	6
27	S0642	Pig	NB	t108	414	3	999	3	3	2	6
28	S0643	Farmer	NB	t108	414	3	999	3	3	2	6
29	S0652	Pig	NB	t108	414	3	999	3	3	2	6
30	S0653	Farmer	NB	t108	414	3	999	3	3	2	6
31	S0662	Pig	Gelder	t108	414	3	999	3	3	2	6
32	S0663	Pig	Gelder	t108	414	3	999	3	3	2	6
33	S0664	Farmer	Gelder	t108	414	3	999	3	3	2	6
34	S0634	Farmer	Utr	t011	426	3	999	3	2	2	7
35	S0644	Pig	Utr	t011	426	3	999	3	3	2	7
36	S0645	Pig	Utr	t011	426	3	999	3	3	2	7
37	S0646	Pig	Utr	t011	426	3	999	3	3	2	7
38	S0647	Pig	Utr	t011	426	3	999	3	3	2	7
39	S0648	Pig	Utr	t011	426	3	999	3	3	2	7
40	S0649	Pig	Utr	t011	426	3	999	3	3	2	7
41	S0650	Pig	Utr	t011	426	3	999	3	3	2	7
42	S0651	Pig	Utr	t011	426	3	999	3	3	2	7
43	S0655	Pig	Gelder	t899	416	3	999	5	3	2	5
44	S0656	Pig	Gelder	t899	416	3	999	5	3	2	5
45	S0657	Pig	Gelder	t899	416	3	999	5	3	2	5
46	S0659	Pig	Gelder	t899	416	3	999	5	3	2	5
47	S0660	Pig	Gelder	t899	416	3	999	5	3	2	5
48	S0661	Pig	Gelder	t899	416	3	999	5	3	2	5
49	S0654	Pig	Gelder	t1939[d]	415	3	999	5	3	2	4
50	S0658	Pig	Gelder	t899	427	3	999	4	3	2	5

[a]: Province where isolates were taken from. NB: Noord-Brabant; Gelder: Gelderland; Utr.: Utrecht.
[b]: SIRU: Staphylococcal Interspersed Repeat Unit; values are the number of repeats (RU); 999: no SIRU PCR amplification.
[c]: RUs of SIRU13 calculated from the lower fragment of the double bands obtained by SIRU13 PCR.
[d]: New *spa*-type observed in this study.

Structure analysis of double fragment of SIRU13.

The genomic region surrounding the SIRU13 was analyzed by DNA sequencing of the pig-related isolate S0835 and the human-derived MSSA ST398 isolate CV55, which also shows the double amplification product. The data were compared with sequence data from completed *S. aureus* genomes. The *fabG* gene is in front of the SIRU13 repeats

At 4 positions silent mutations were observed. At position 315 RF122, a bovine MRSA, shows a G to A mutation; at position 363 C to C change occurred in 3 of the 18 isolates; at position 412 a C was substituted for a T in 12 isolates; at position 553 all isolates had a T except S0385 and CV55. A single amino acid change (leucine to proline) was observed for S0385 due to the presence of a T instead of a C at nucleotide position 449. This result was confirmed by Sanger sequencing. Downstream of the SIRU13 region the *acpP* gene is located, which is also known as *hmrB*. No mutations were observed in this gene. The gene structure between these 2 genes is highly variable and unusual (Fig. 2).

The number of repeats R is variable. All isolates have R1, but this repeat is composed of only the first half the other repeats (Fig. 3). All isolates except RF122 have a sequence called D1 upstream of the variable number repeats. The forward primer for SIRU13 is located in this sequence. In MW2, the closely related MSSA476, S0385, and CV55 another copy of a SIRU13 repeat (R0) is located upstream of D1. In S0385 and CV55 another copy of D1 (called D2) is present in front of R0. In all isolates a sequence indicated as P1 is present between the D and R sequences. This sequence P1 shows strong resemblance to the last part of the R sequences (Fig. 3). Two sequence variants were observed.

The sequences of R1 of the different isolates are identical. However, 13 sequence variants were observed that belonged to 3 subgroups based on the highly divergent sequences between nucleotides 30 and 46 (Fig. 3). Sequence

variation is also observed at other positions particularly at the end of the repeats. Three sequence motifs are found: CAAGA, CATGCA and AA. These motifs are not correlated with the subtypes. Two of the motives divide the P1 sequences in two groups (Fig. 3).

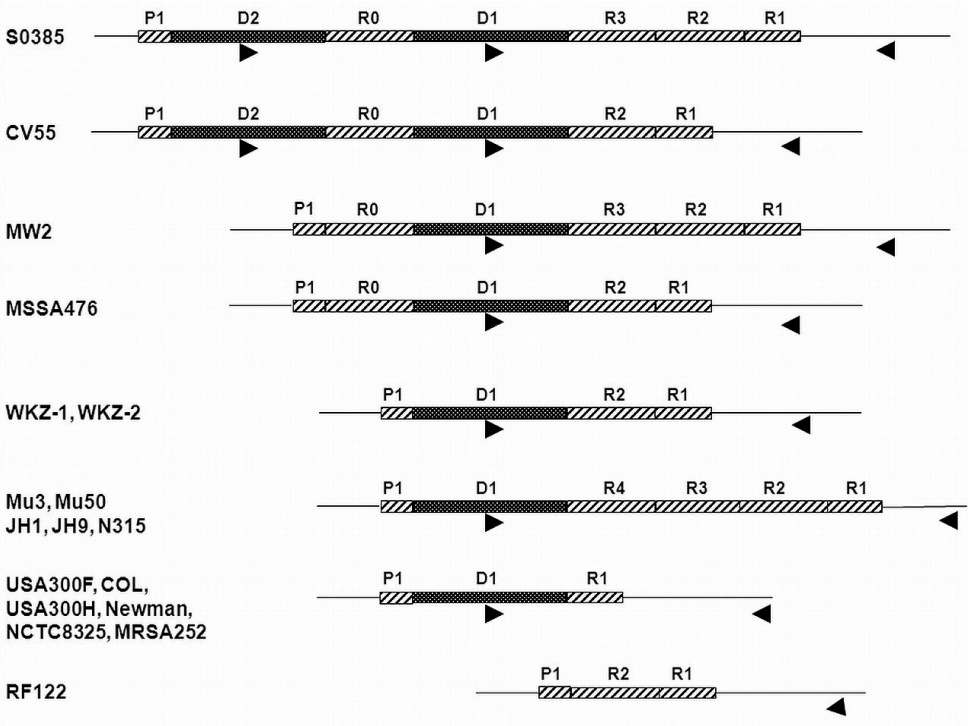

Figure 2. Comparison of the SIRU13 regions of 18 sequenced *S. aureus* genomes. Sequences with similarity have identical shading. SIRU13 repeats are denoted as R. P1 denotes a small sequence partially present in the repeats. D denotes sequences that may be duplicated in some strains. The thin line denotes conserved chromosomal sequences. For details see the text.

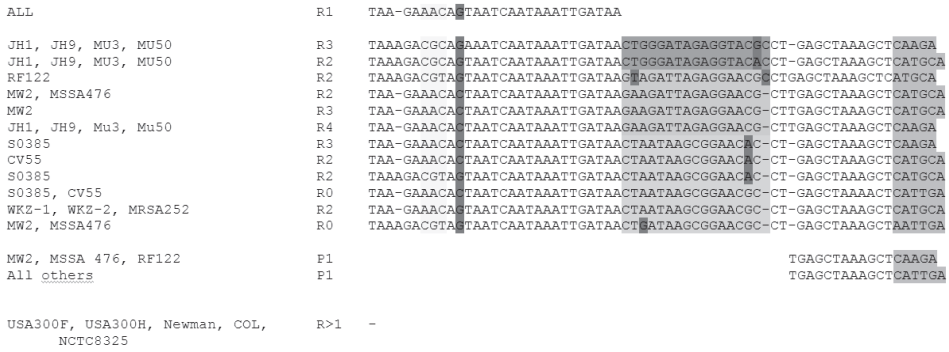

Figure 3. Comparison of the repeat sequences of SIRU13. Also included the sequences of P1. Colors and gray indicate variable sequences. A hyphen indicates a lacking nucleotide.

The repeats are not exact duplicates in isolates with multiple complete repeats with the exception of MW2. In isolates JH1, JH9, Mu3 and Mu50 with 3 complete repeats all repeats are different and even belong to two different subtypes. With the exception of WKZ-1 and WKZ-2 the MRSA-MSSA pairs show a repeat difference. The MRSA strains MW2 and S0385 have an additional repeat compared to their related MSSA strains MSSA476 and CV55, respectively. Mutations do not correlate with MRSA or MSSA.

The sequence between the repeat and the start of *acpP* is highly conserved. It showed only a C to G mutation in the first nucleotide after R1 in 12 strains and an A to G mutation in 5 strains, 51 nucleotides closer to the *acpP* gene.

Analysis of potential secondary structure in an RNA folding program shows that isolates with different repeat numbers show different structures (Fig. 4). So, the SIRU13 region is a highly variable and complex sequence.

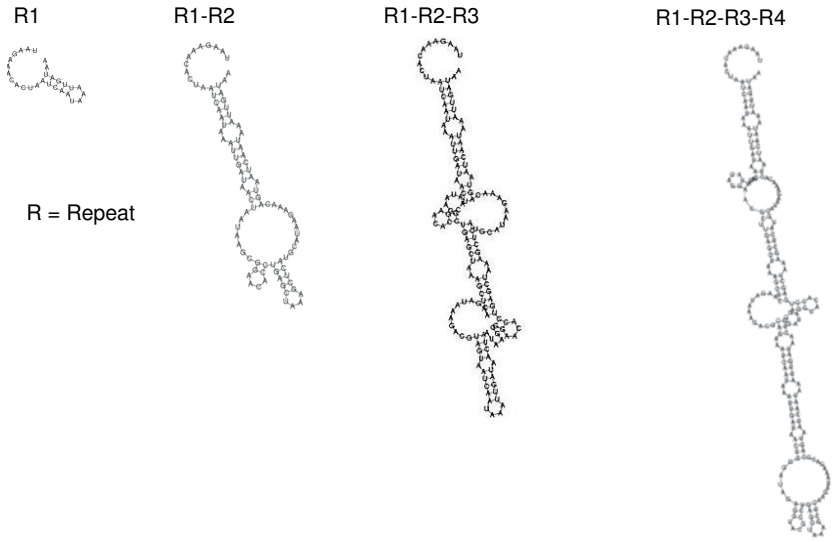

Figure 4. Secondary structures possible in the repeat regions of SIRU13 depending on the repeat number.

DISCUSSION

MLVA has demonstrated great potential for fast and reliable typing of human and bovine *S. aureus* isolates. A previous study demonstrated that a newly developed MLVA scheme for human *S. aureus* had a higher discriminatory power than MLST, *spa*-typing, and MLVA with Simpson's indices of diversity of 0.941, 0.963, and 0.987, respectively. While for bovine mastitis *S. aureus*, the Simpson's indices of diversity were 0.887, 0.831, 0.69, and 0.781 for PFGE, MLST, *spa*-typing and MLVA, respectively, indicating that discriminatory power of MLVA was between MLST and *spa*-typing, whereas PFGE displayed the highest discriminatory power. In this study, although all isolates tested belonged to the same sequence type (ST398), MLVA displayed higher discriminatory power than *spa*-typing and very well predicted the *spa*-types. Almost all isolates belong to the *spa*-types that are commonly associated with ST398 (t011, t108, t567, and t899). In addition, an isolate belonging to the MT 415 had a new *spa*-type (t1939) with single locus variation compared to the MT 416 isolates. This might suggest that t1939 is a pig-related *spa*-type.

In this study, none of the fifty isolates were positive for SIRU05. Previously we reported the absence of SIRU05 in *S. aureus* originating from bovine mastitis.

Sequencing of the region neighboring SIRU05 showed replacement of a fosfomycin resistance element by insertion sequence IS*RX* , which explained the negative result of SIRU05 PCR (8). It is likely that the absence of the fosfomycin resistance element also explains the absence of amplification products for pig-related *S. aureus*.

An unexpected result was that amplification of SIRU13 yielded two fragments in all isolates. In a previous study, we included two pig-related *S. aureus* isolates collected from humans, which also showed a double fragment in the SIRU13 PCR. These isolates had MLVA profiles 413 and 426, and *spa*-type t011 and t567, which are identical to that of isolates in this study (9). The size difference between the two fragments was the same for all isolates. This is supported by the sequence data of the fragments. Therefore the number of RUs of SIRU13 was calculated based on the lower fragment.

The highly variable and complex sequence in the intergenic region of the *fabG* and *acpP* gene, both possibly involved in acyl metabolism, suggests that this region is involved in regulation (Fig. 2). Furthermore, secondary structures can be easily formed in this region and the details of these structures differ with differing repeat units (Fig. 4).

Within the repeats the majority of nucleotides are conserved suggesting conserved protein or RNA binding domains (Fig. 3). Also the sequences of the D1 sequences are strongly conserved allowing only a few mutations (Fig. 3). Because the repeats are approximately 63 bp long variation in repeat number is most likely generated by recombination. This is further supported by the observation that in some isolates like JH1, JH9, Mu3, and Mu50 repeats are not identical and even belong to different subtypes that cannot arise by simple mutation. The one repeat difference between the related MSSA MRSA pairs MW2/MSSA476 and S0835/CV55 suggests that this difference is related to the difference in susceptibility for methicillin. However, WKZ-1/WKZ-2, which are also a MRSA/MSSA pair do not show the one repeat difference. This may be explained by the fact that both isolates were obtained only a few weeks apart and full adaptation has not occurred (21).

In favor of the hypothesis that the SIRU13 region is involved in regulation of methicillin resistance is, that is located upstream of the *hmrB* gene. This gene was identified by Kondo et al to induce Eagle-type methicillin resistance when

cloned into *S. aureus* N315, an MRSA with low *mecA* expression due to regulation by an intact *mecI* regulator locus and possibly conversion of heterogeneous to homogenous expression of methicillin resistance (11). The fact that the WKZ-1/WKZ-2 pair does not show a difference is not necessarily contradictory with the hypothesis that *hmrB* and thereby methicillin resistance is regulated by SIRU13. The first explanation may be that the MRSA was captured only shortly after its supposed generation. The second explanation is that the conversion of heterogeneous to homogeneous methicillin expression is caused by a different mechanism. Kondo et al showed that overexpression of another gene, termed *hmrA*, resulted in the same phenotype as *hmrB* overexpression (11). This gene shows homology with an amidohydrolyse, but a possible role in methicillin resistance is not clear.

Summarizing we can say that the MLVA scheme developed for typing human *S. aureus* isolates appears also to be applicable to pig-related MRSA. In pig-related ST398 isolates a 162 bp duplication has taken place in SIRU13. This region is highly variable between different isolates and may be involved in regulation of methicillin resistance.

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Chapter 5

Virulence factors of genotyped bovine mastitis *Staphylococcus aureus* isolates in The Netherlands

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Submitted

ABSTRACT

Virulence genes required for bovine mastitis are not well defined. The objective was to investigate the virulence factor profiles among extensively genotyped bovine mastitis *S. aureus* isolates from The Netherlands. A total of 76 *S. aureus* isolates from 2 institutions that represented regional and national strains were examined by PCR for 19 toxin genes, 12 adhesin genes, 3 immune evasion genes, and 6 other genes. The presence of *hlb*, *ebps*, *efb*, *sspA* and *sspB* was observed in all isolates, *coa* in all isolates but one, while the presence of *sec3, seg, seh, sei, sel, sem, sen, tst*, *lukE, fnbA*, *fnbB*, i*caB*, *icaC*, *icaD*, *clfA, sdrE*, *cna, cap8*, *cap5,* and *map* was variable. *Sak* and the genes for the human specific immune evasion proteins, SCIN and CHIPS were detected in only one isolate. In addition, none of isolates were *lukPV*-positive. Five major virulence factor profiles were observed which, with the exception of SaPIbov, were in general agreement with the clustering obtained from PFGE, MLST, *spa*-typing and MLVA.

1. INTRODUCTION

Virulence of microorganism can be defined as the capacity of the microorganism to damage the host and that may lead to signs and symptoms of diseases. Pathogenic microorganisms are characterized because they produce virulence determinants. Virulence factors have been extensively studied in order to better understand the way microorganism induce diseases. Not every encounter with pathogenic microorganism leads to disease. That depends on the virulence factors, the host defense mechanism and the genetic deposition of the host. *S. aureus* infection starts with successful colonization of both human and animals, the defense mechanism of the host and the virulence factors produced by the *S. aureus*.

S. aureus may produce a number of exotoxins including the classical staphylococcal enterotoxins (SEs) A to E, the recently characterized SE types (SEG-SEU) [1;2], toxic shock syndrome toxin 1 (TSST-1), β-hemolysin (*hlb*), the bi-component Panton-Valentine Leukocidin (Luk-PVL), proteases with the ability to induce T-cell proliferation, and exfoliative toxin A and B (ETA and ETB). A wide array of cell surface adherence proteins belong to the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which promote adhesion to host extra cellular matrix components, such as fibrinogen, fibronectin and collagen [3]. Clumping factor A and B (ClfA and ClfB) are two dominant fibrinogen-binding adhesins [3], while fibronectin-binding protein A and B (FnBPA and FnBPB) mediate binding to fibronectin, and CNA binding to collagen. Serine-aspartate repeat protein (Sdr) are surface proteins related to ClfA and ClfB [4] which have been hypothesized to mediate interactions of *S. aureus* with the extra cellular matrix [5]. Coagulase induces polymerization of fibrinogen to fibrin.

Distinct from coagulase, fibrinogen-binding protein (Efb) is an intracellular protein which specifically promotes binding to fibrinogen [6]. Elastin-binding protein of *S. aureus* (EbpS) mediates binding to soluble tropoelastin [7]. Adherence of *S. aureus* is also enhanced by a MHC class II analog protein (MAP), a protein with broad binding activity to fibrinogen, collagen, fibronectin, vitronectin, and elastin which may enable colonization of different tissues. Plasmin-sensitive protein (Pls) has an adhesive function by mediating interaction between bacterial cells, bacterial binding to immobilized fibronectin and immunoglobulin G, invasion of epithelial cells, as well as anti-adhesive functions at a certain stage during

infection which allow the bacteria to spread. *S. aureus* also is an important cause of device-associated infections due to biofilm formation mediated by gene products of the *icaADBC* locus [8]. *S. aureus* exoenzymes including serine protease (SspA) and cysteine protease (SspB) are extra cellular proteases involved in elastin degradation known as elastinolytic activity.

Capsular polysaccharide (CP) produced by *S. aureus* renders the bacteria resistant to phagocytosis, so they can persist in the bloodstream. Eleven CP serotypes have been described and CP type 5 and 8 were predominantly found in clinical isolates [9;10;11]. Furthermore, *S. aureus* phages encoding immune evasion molecules such as staphylokinase (SAK), chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) and staphylococcal complement inhibitor (SCIN) may provide *S. aureus* the ability to escape the human immune system. In addition, an arginine catabolic mobile element (ACME) which inhibits polymorphonuclear cell production, is considered to enhance the virulence and colonization by *S. aureus* [12].

In veterinary medicine, *S. aureus* is generally recognized as the most common pathogen of contagious bovine mastitis and it causes significant economic losses in the diary industry. Chronic and deep infections in the mammary glands of bovines are often associated with poor success of treatment. Different suites of virulence factors may result in differences in severity and course of mastitis. However, little is known about the presence of *S. aureus* virulence genes in bovine mastitis and so far, dominant virulence factors among bovine mastitis *S. aureus* have not been identified. In this study, we investigated the virulence factor profiles from different genotyped clinical bovine mastitis *S. aureus* isolates from The Netherlands.

2. METHODS

2.1. Bacterial isolates

Seventy six *Staphylococcus aureus* isolates from clinical cases of bovine mastitis were included. Thirty isolates were obtained by the Faculty of Veterinary Medicine Utrecht University, Utrecht, The Netherlands, from 26 farms near Utrecht. Isolates

from farms sampled twice were taken at least one year apart. Another 46 isolates were collected by the Animal Health Service in Deventer, The Netherlands for the Central Veterinary Institute (CVI), Lelystad, The Netherlands from farms throughout The Netherlands. The sources were individual teat milk samples from dairy cattle from all over the country from clinical or subclinical cases of mastitis. Each isolate from CVI represents one farm, the location of the farms is unknown but they are distributed all over the Netherlands. All isolates were methicillin-susceptible *S. aureus* (MSSA) and collected between 1988 and 2005.

These isolates were genotyped by Multilocus Sequence Typing (MLST), staphylococcus protein A (*spa)*-typing, Multiple Locus Variable Number Tandem of Repeat (VNTR) Analysis (MLVA) and Pulsed-Field Gel Electrophoresis (PFGE) [13]. Most of the 76 bovine *S. aureus* isolates used in this study belonged to 4 dominant MLST types (STs) ST 71 (n=7), ST 151 (n=11), ST 479 (n=21), ST 504 (n=21). Further, 10 new STs were detected in 13 isolates, ST97 in 2 isolates, and one singleton. We observed 3 dominant *spa*-types, t524 (n=13), t543 (n=22) and t529 (n=36), 5 isolates with unique *spa*-types including 2 new *spa*-types, t2112 and t2248. MLVA typing showed 2 dominant MLVA types (MTs), MT102 (n=32) and MT112 (n=17), 6 isolates of MT118, 4 isolates of MT114 and 125, and 13 isolates having a unique MT. DNA of 76 isolates was digested with *Sma*I and showed 21 PFGE profiles, 4 dominant profiles, 10 profiles related to the dominant profiles and 7 unique PFGE profiles (Figure 1). No particular difference was observed between regionally and nationally obtained *S. aureus* bovine mastitis strains in terms of PFGE profile, MLST and *spa*-types, except for MT 118 that was only present among regionally obtained isolates. Despite the fact that isolates could belong to the same cluster, they were not considered to belong to local outbreaks as at least 90% of the isolates were obtained on different farms whereas the remaining isolates were obtained at least one year apart when sampled on the same farm. A minimum spanning tree based on MLST generally showed a distinct cluster of bovine *S. aureus* compared to isolates from the human population, although little sharing of strains between the bovine and human population was observed. In addition, the ST or clonal complexes (CC) of bovine mastitis isolates from The Netherlands differed from those described elsewhere with the exception of 4 isolates (S0337, S0426, S0431 and S0436) that belonged to CC97 which has previously been reported to be obtained from humans.

2.2. Genomic DNA preparation

The isolates were grown on blood agar (Trypticase soy agar II containing 5% sheep blood) overnight at 37°C prior to DNA isolation. Preparation of bacterial genomic DNA was performed using the NucleoSpin kit (Macherey-Nagel) following the protocols from the manufacturer with the exception that the bacterial pellet is resuspended in 20mM Tris/HCl, 2 mM EDTA, and 1% Triton X-100 supplemented with 0.2 mg/ml of lysostaphin, achromopeptidase and RNase.

2.3. PCR amplifications

PCRs were performed to detect 19 staphylococcal toxin genes including: 13 enterotoxin genes: *sea*, *seb*, *sec3*, *seg*, *seh*, *sei*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, *seq*, *tst*, *lukE*, *lukS/F*, *eta*, *etb*, and *hlb*; 12 adhesion genes: *fnbA*, *fnbB*, *clfA*, *efb, cna*, *sdrE*, *icaB*, *icaC*, *icaD*, *ebpS*, *map*, and *pls*; 3 genes encoding extra cellular proteases: *coa, sspA* and *sspB*; 3 immune evasion genes: *sak*, *chips* and *scin*; 2 genes for capsular polysaccharide type 5 and 8 production: *cap5a* and *cap8*; and SCC_{ACME}. Table 1 lists the oligonucleotide primers, positive and negative control strains used in this study. PCR was performed in 25 µl containing HotStarTaq Master Mix (QIAGEN), 25 pmol/ml of each primer and genomic DNA. The PCR product was analyzed on 1% agarose gel stained with ethidium bromide and visualized under UV-light.

Table 1. Oligonucleotide primers used. UMCU: Primers were designed at Dept. of Medical Microbiology, University Medical Centre Utrecht, Utrecht, The Netherlands.

Gene	Primer sequence (5'-3')	Positive Control (1)	Negative Control(2)	Reference (3)
Toxin genes				
sea	ttaaccgaaggttctgtaga	MSSA476	COL	[22]
	agatcattcgtggtataacg			
seb	tacacccaacgttttagcagaga	COL	-	[24]
	aaggcgagttgttaaattcatagagt			
sec3	aaaaattatgacaaagtgaaaacagagt	Mu50	COL	[25]
	gcaggcatcatatcataccaaaaagt			
seg	aactatgggaaatgtaatgaatctt	MRSA252	MSSA476	UMCU
	gccagtgtcttgctttgtaatc			
seh	cacatcatatgcgaaagc	MSSA476	-	UMCU
	cgaatgagtaatctctagg			
sei	atctaataattgggacgaa	MRSA252	COL	UMCU
	aaaaacttacaggcactc			

Gene	Primer sequence (5'-3')	Pos. Ctrl.[1]	Neg. Ctrl.[2]	Ref[3]
sek	tactcctatagctaatcaact	USA 300	-	UMCU
	ggtaacccatcatctcc			
sel	taacggcgatgtaggtccag	N315	COL	UMCU
	attttgaagaagtgccgtatt			
sem	ggataattcgacagtaac	N315	MSSA476	UMCU
	tttcagtttcgacagttt			
sen	tatatccgtacttaaaacttcta	N315	MSSA476	UMCU
	aaaaactctgctcctactg			
seo	agaccctattgctttaca	N315	MRSA252	UMCU
	tctttatgctccgaatga			
sep	aatcataaccaaccgaatca	N315	MSSA476	[22]
	tcataatggaagtgctataa			
seq	tctagcatatgctgatgtagg	MRSA252	MSSA476	[26]
	caatctcttgagcagttacctc			
hlb	gttggtgcacttactgacaa	COL	-	[22]
	tgtgtaccgataacgtgaac			
tst-1	attttacccctgttcccttatcatc	Mu50	MSSA476	UMCU
	taggtggtttttcagtattgtattca			
luk-E	gcaactttgtcagtaggactg	COL	MSSA476	UMCU
	gtctacttcactgacataactc			
luk-S/F	cacagtggtttcaatccttc	USA 300	-	UMCU
	cacctgataagccgttagag			
eta	gttggtgcacttactgacaa	COL	-	UMCU
	tccacggatttttatttttatttattac			
etb	atattatttttacacccgctcaa	S0202	-	UMCU
	ttccccaaagtgtctccaaaagta			
efb	cgaaggatacggtccaagag	COL	-	UMCU
	ctgccttttgtgcttttctg			
Adhesion genes				
clfA	caactttgaccatgcccgcttat	COL	MSSA476	UMCU
	ccaggctcatcaggttgttcagg			
cna	taaaacgggagatatgctaccag	MSSA476	USA300	[27]
	gatttcccgttcacttcttcctta			
coa	atattattagccgttacaggtg	COL	-	[28]
	aatgcgcgtttatcttga			

Gene	Primer sequence (5'-3')	Pos. Ctrl.[1]	Neg. Ctrl.[2]	Ref[3]
ebpS	atgtatttgcttggttag	N 315	COL	[8]
	aaatcgtcaatcgataga			
fnbA	attgggagcagcatcagtattcttagga	COL	-	UMCU
	gatcttgtcacacgtggcttactttctg			
fnbB	taaagcaagcgaaacacaaacaactac	COL	MRSA252	UMCU
	atctcccgccttaattccttctc			
icaB	ttgatcatattgcctgtaagc	MSSA476	-	[29]
	aatcgtgggtatgtgtttca			
icaC	atggtatggctattttatcgttgtta	MSSA476	-	UMCU
	gtgatatcgctagaaagaccattgta			
icaD	atcgctatatcttgtgtcttt	MSSA476	-	UMCU
	tctctgccatttttgaa			
cap5a	aagtggtgggagaaaacgcctgat	N315	MRSA252	UMCU
	ttcttttgctgcatgacttactgttacg			
cap8	atgacgatgaggatagcg	MW 2	COL	UMCU
	cacctaacataaggcaag			
map	tagaggtatcggggaacg	COL	MRSA252	UMCU
	aatctttttaatatctttggcacta			
pls	caagttggtcaaaatgccctaaata	COL	MRSA252	UMCU
	ttcaccacgttcaactacatctcct			
sspA	ttattgcttccggtgtagttgtag	COL	-	UMCU
	atctgggttattaggttggtcatc			
sspB	agaagatggcaaagttagattagt	COL	-	UMCU
	tgtgggtcattagggttttg			
sdrE	atcaaagttggcgatggt	Newman	MRSA252	UMCU
	ccatggaccattggatct			
Immune evasion and other proteins				
sak	aaggcgatgacgcgagttat	MRSA252	-	[22]
	gcgcttggatctaattcaac			
scin	agcacaagcttgccaacatcg	MRSA252	-	[22]
	ccggaattcttaatatttacttttagtgc			
chips	gaaaaagacattagcaacaacag	MRSA252	-	[22]
	cataagatgatttagactctcc			
SCC$_{ACME}$	gaatgaacgtggatttaatgtcc	USA300	MRSA252	UMCU
	cgttatggaggtgctctg			

3. RESULTS

3.1. Detection of virulence factor encoding genes

3.1.1. PCR amplification of staphylococcal toxin and protease genes

Among the 18 toxin genes tested, *hlb*, *sspA*, and *sspB* were detected in all isolates. The 9 genes for enterotoxins A (*sea*), B (*seb*), K (*sek*), O (*seo*), P (*sep*), Q (*seq*), leukocidin encoded by *LukS/F*, exfoliative toxin A (*eta*) and B (*etb*) could not be amplified while the 8 other genes were present in 1-80% of the isolates. The recently described enterotoxins G and I were the most prevalent (78% and 80% of the isolates, respectively). We observed that 56 of 76 (76%) isolates encoded both SEI and SEG. Forty (52.6%) isolates encoded SEM and SEN, although these two genes were not present simultaneously in 27 (35.5%) isolates. The *seh* gene was present in only 1 isolate (1%). The SaPIbov pathogenicity island signature combination of *sec3*, *sel*, and *tst* was found in 15 (19.7%) isolates. Two (2.6%) isolates encoded *sec3* and *tst* but lacked *sel,* while 6 (7.9%) other isolates encoded either *sec3*, *sel* or *tst* only. In addition, *lukE* was detected in 20% of isolates.

3.1.2. PCR amplification of genes encoding staphylococcal proteins for adhesion

The *ebps*, and *efb* could be amplified for all isolates, while *pls* was absent. PCR of *fnbA* and *fnbB* showed that 73 (96%) isolates possessed *fnbA* and 33 (43%) isolates had *fnbB*. Three gene targets involved in biofilm formation, i*caB*, *icaC* and *icaD*, were found in 64 (84%), 63 (83%) and 65 (86%) isolates, respectively. *ClfA* was detected in 16 (21%) isolates, 33% (n=16) isolates were positive for *sdrE*, 49% (n=37) were *cna* positive, 18% (n=14) contained *map*, and most of isolates (99%, n=75) obtained *coa.*

3.1.3. PCR of genes encoding immune evasion molecules and other S. aureus protein.

The three immune evasion genes, *sak, scin* and *chips*, could not be amplified in almost all isolates, except for one isolate (S0336). SCC_{ACME} was not amplified in any of the isolates. *cap8* detected in 73 isolates, while *cap5* obtained in 3 isolates.

3.2. Distribution of virulence factors according to different typing methods

Five main clusters (A, B, C, E and F) were identified among the 76 bovine mastitis *S. aureus* isolates based on the presence and absence of virulence factors (Figure 1). In addition, a unique gene pattern (called D) was observed in 1 isolate (S0366). There is general agreement between the four typing methods used (PFGE, MLST, *spa*-typing and MLVA) and the 6 clusters of isolates, but exceptions were obviously present. Cluster B and C could not be identified based on PFGE profiles, since some of the isolates with an identical PFGE profile were distributed across both virulence profile clusters. These isolates were also considered closely related based on the other typing methods (MLST, *spa*-typing and MLVA).

Cluster A isolates mostly possessed *sdrE*, *finbB*, and *cna* genes which were totally absent in cluster B and C isolates, and occasionally present in cluster D and E isolates (Table 2). Identical and closely related isolates identified by MLST, *spa*-typing and MLVA in cluster B and C had similar virulence gene patterns, except that SaPIbov (*tst, sec3* and *sel*) were predominantly detected in cluster C isolates. Virulence gene patterns detected in cluster E and F isolates mostly lack of SaPIbov similar to isolates of cluster A. The gene for enterotoxin *sem* was absent, while *sen*, *seg*, and *sei* were occasionally detected. Almost all isolates from both clusters contained *lukE, clfA*, and *map*, while other clusters did not. *icaB-D* and *cap8* were detected in all clusters (except cluster E isolates. The only isolate in cluster D showed a unique gene pattern with the presence of *seh* and a unique combination of other virulence genes.

Thus in general, there is an agreement between the virulence profile and the typing methods and the clusters with exception of clusters B and C. Cluster C contains SaPIbov-positive isolates, whereas this is absent in cluster B isolates, but the overall virulence gene profiles are similar for both clusters.

By analyzing the presence of all virulence factors tested in all isolates, a minimum set of genes is likely required for bovine mastitis. *FnbA, hlb, ebps, sspA, sspB* and *coa* were always present in Dutch isolates, while *cap8*, *sem, seg, sei, icaB, icaC, icaD* and *sen* were variable present.

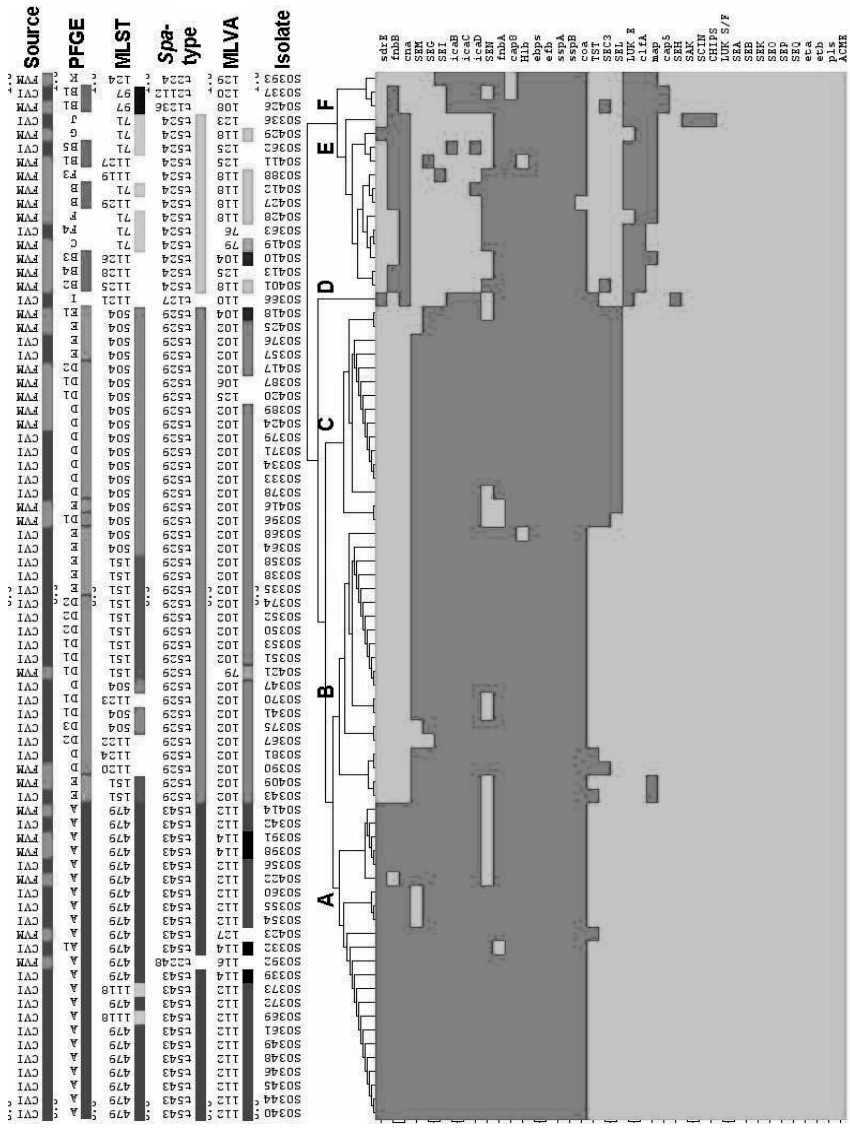

Figure. Virulence factor profiles observed within various typing methods.
Source: Place where the isolates were taken. FVM: Veterinary Faculty Utrecht University; CVI: Central Veterinary Institute, Lelystad, The Netherlands.
The presence and absence of genes indicated by red and green spots.
Virulence factor profiles were defined based o the presence and absence of the genes, and resulted 5 main profiles, A, B, C, D, and E.

Table 2. Presence and absence of toxin and adhesion gene profiles in different bovine PFGE-types.

Vir. gene profile[a]	PFGE profiles (n)	*hlb*[b]	SaPI bov[c]	*egc*[d]	Other toxin genes	CT[e]	*clfA*[f]	Adhesin genes present
A	A (13)	(+)	(-)	(+)	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A1 (1)	(+)	(-)	(+)	(-)	8	(-)	*fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (1)	(+)	*tst*	(+)	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (3)	(+)	(-)	*seg, sei, sen*	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (4)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, fnbB, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
A	A (1)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, sdrE, cna, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	E (1)	(+)	*tst*	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
B	E (1)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
B	D2 (1)	(+)	(-)	*sei, sen*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D3 (1)	(+)	(-)	*seg, sei, sen*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D (1)	(+)	*sec3, tst*	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D (1)	(+)	*tst*	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D1 (2)	(+)	(-)	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	D (1), D1 (3), D2 (3), E (4)	(+)	(-)	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
B	E (1)	(-)	(-)	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	D1 (1)	(+)	*sec3, tst*	*seg, sei, sem*	(-)	8	(-)	*ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	E (1)	(+)	(+)	*seg, sei, sem*	(-)	8	(-)	*ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	D (1)	(+)	(+)	*seg, sei, sem*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	D (6), D1 (2), E (2)	(+)	(+)	(+)	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*

Vir. gene profile[a]	PFGE profiles (n)	*hlb*[b]	SaPI bov[c]	*egc*[d]	Other toxin genes	CT[e]	*clfA*[f]	Adhesin genes present
C	E (1)	(+)	(+)	*seg, sei, sen*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
C	E1 (1)	(+)	(+)	*seg, sei*	(-)	8	(-)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, coa*
D	I (1)	(+)	*tst*	(-)	*seh, lukE*	8	(-)	*fnbA, sdrE, cna, ebps, efb ,icaB, icaC, icaD, sspA, sspB, map, coa*
E	B3 (1)	(+)	(-)	(-)	*lukE*	8	(+)	*fnbA, cna, ebps, efb, sspA, sspB,map, coa*
E	C (1), F4 (1)	(+)	(-)	*sen*	*lukE*	8	(+)	*fnbA, cna, ebps, efb, sspA, sspB, coa*
E	F (1)	(+)	(-)	*sen*	(-)	8	(+)	*fnbA, cna, ebps, efb, sspA, sspB,map, coa*
E	B2 (1)	(+)	*sec3*	*sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, coa*
E	B4 (1)	(+)	(-)	(-)	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, coa*
E	B (1)	(+)	(-)	*sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map*
E	B (1)	(+)	(-)	*sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, icaD, sspA, sspB, map, coa*
E	F3 (1)	(+)	(-)	*sei, sen*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map, coa*
E	B1 (1)	(-)	(-)	*seg*	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map, coa*
E	B5 (1)	(+)	(-)	(-)	*lukE*	8	(+)	*fnbA, fnbB cna, ebps, efb,icaB, icaD, sspA, sspB, map, coa*
E	G (1)	(+)	(-)	(-)	(-)	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB,sdrE, map, coa*
E	J (1)	(+)	(-)	(-)	*lukE, sak, scin, chips*	8	(+)	*fnbA, fnbB cna, ebps, efb, sspA, sspB, map, coa*
F	B1 (1)	(+)	*sec3*	*sen*	*lukE*	5	(+)	*fnbA, fnbB, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
F	B1 (1)	(+)	(-)	(-)	*lukE*	5	(+)	*fnbA, fnbB, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*
F	K (1)	(+)	(-)	*sei, sen*	*lukE*	5	(+)	*fnbA, ebps, efb, icaB, icaC, icaD, sspA, sspB, map, coa*

[a]: Virulence gene profile.
[b] : β-hemolysin.
[c] : Bovine staphylococcal pathogenicity island characterized by the presence of *sec*, *sel*, and *tst*. The (+) means *sec*, *sel* and *tst* genes were amplified, while the (-) means none of the genes were detected. Written gene/s depicted their presence.
[d]: Enterotoxin gene cluster (*egc*) consists of 5 SEs, *seg, sei, sem, sen*, and *seo*. Since all of our isolates lack of *seo*, the (+) indicated the presence of *seg, sei, sem* and *sen*.
[e]: Capsular type.
[f]: Clumping factor A.
n: Number of isolate.

4. DISCUSSION

The presence of virulence factor encoding genes and variation in their presence in extensively genotyped *S. aureus* isolates collected from bovine mastitis was investigated. *Hlb*, *ebps*, *efb*, *sspA* and *sspB* were found in all isolates tested and *coa* in all isolates except one, *while sea*, *seb*, *sek*, *seo*, *sep*, *seq*, *PV- LukS/F*, *eta*, *etb, pls*, and SCC_{ACME} were completely absent. Variation in presence was shown for 9 toxin genes: *sec3, seg, seh, sei, sel, sem, sen, tst*, and *lukE* and 9 adhesion related genes: *fnbA*, *fnbB*, i*caB*, *icaC*, *icaD*, *clfA, sdrE*, *cna,* and *map*, and other genes such as *scin, chips*, *cap8* and *cap5*. From these data a minimum set of genes necessary for *S. aureus* bovine mastitis was distilled which includes *hlb, efb, ebps, sspA, sspB,* and *fnbA.* However, *efb* has been reported to be present in only part of the isolates by others [14]. This does not exclude that other virulence factors do not contribute to the severity of disease. An alternative explanation is that different gene profiles may encode factors required to cause bovine mastitis. However, details of the pathogenesis of bovine mastitis may then differ for different gene profiles.

The virulence factor gene profiles in general agreed with the clustering obtained from the 4 genotyping methods. The exception of cluster B and C was caused by the presence of SaPIbov in a part of the isolates belonging to PFGE types D, D1, D2, and E. This result indicates that SaPIbov was acquired independently by several isolates belonging to different genetic lineages. The clustering of *tst*, *sec3*, and *sel* genes (here reported in 19.7% of the isolates) is specific for a bovine pathogenicity island known as SaPIbov [1]. 10.5% of all isolates carried either 1 or 2 genes representative for SaPIbov. The *tst* gene has also been reported on other pathogenicity islands [15]. Whether the presence of *sec3* and *sel* in two isolates and *sec3* or *sel* in other isolates implicates that SaPIbov is incomplete or that the *sec3* and *sel* can be present on other locations remains to be determined. Although SaPIbov may contribute to mastitis it not required for disease or spread of bovine isolates between animals, because other lineages appear to be equally widespread as cluster C (Figure).

The presence of *hlb, coa* in nearly all isolates is in agreement with other reports [2;14]. The genes that were not identified among Dutch bovine mastitis isolates were also generally absent from isolates from other countries with the exception

of *sea*, *sek*, *seo* and *eta* and *etb* [9;14;16-18]. *Sea* and *seo* for example have been reported to be present in 77% and 52% of mastitis isolates in Switzerland [16]. The variability of the presence of other toxin genes reported here is in general agreement with other reports [14;16-19]. The coexistence of the toxin genes *seg* and *sei* has been described before and further explained by the presence of both genes in a tandem orientation belong to an operon of enterotoxin gene cluster (*egc*) [20]. In addition, we detected *sem* and *sen* within *seg-* and *sei-* harboring *S. aureus* isolates without the presence of *seo*. This finding is in contrast to those studies demonstrated *sem*, *sen, seg, sei* and *seo* predominate in *S. aureus* from animal hosts[1;17]. None of our isolates were *lukPV*-positive in agreement with the study done by Fueyo et al [21], although Zecconi et al identified *pvl* in more than 50% of isolates from bovine mastitis [14]. The reason for this discrepancy is not known, although the number of different genetic clusters observed among the Dutch isolates was low indicated that most isolates were related. However, also among human methicillin-susceptible *S. aureus* only a few percent of the isolates carry the *pvl* genes.

One of the common features of invasive bacterial pathogens is a capsule. In this study, the *cap8* was predominant compared to the *cap5*. Poutrel et al. described the difference in distribution of CP5 and CP8 between clinical *S. aureus* isolates collected from humans and bovines. He observed 51.4% of *S. aureus* bovine origin were CP5 positive and 18% were CP8 positive, while the percentages of human *S. aureus* harboring CP5 and CP8 were 20% and 60%, respectively [10]. Several studies described that CP5 and CP8 were commonly found in the clinical human *S. aureus* isolates. Sordelli et al reported that the geographical region influences the differences in CP5 and CP8 distribution [11]. Since CP 5 and 8 could be recovered from either human or animal derived *S. aureus* isolates, we conclude that obviously CP5 and 8 seems to be non-host specific.

Only one isolate carried the genes for CHIPS and SCIN, which interfere with activation of the human complement system but not the bovine complement system. Both genes are located on the same bacteriophage that is integrated in the hlb gene [22]. The *hlb* product is poorly effective against human erythrocytes, but highly effective against bovine erythrocytes and bacteriophage insertion disrupts the *hlb* gene. Therefore, we consider this isolate as human derived. ST97 isolates have been obtained from both humans and bovines [23]. The gene profile of these isolates resembles the gene profile of bovine mastitis isolates and lack the human complement specific interference factors (Table 2 and Figure).

Based on these data we believe that ST97 *S. aureus* isolates are from bovine origin.

In conclusion, the virulence factor profiles with the exception of SaPIbov were generally in agreement with the typing results. This suggests independent mobilization of this pathogenicity island. Five major virulence factor profiles were observed. The variability in gene content indicates that either a minimum set of genes is required to cause bovine mastitis or that different gene profiles can cause bovine mastitis. The latter option suggests that pathogenesis may differ in detail between different strains. Of the tested virulence genes *hlb, ebps, sspA, sspB, fnbA,* and *coa* seem to compose the minimum gene set necessary for bovine mastitis. We presume that additional virulence factors genes present may contribute the severity of disease.

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Chapter 6

Transmission of methicillin-resistant *Staphylococcus aureus* strains between different kinds of pig farms

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SUMMARY

The main objective of the present study was to investigate if different kinds of pig farms, like farrowing farms and rearing farms, play a role in the transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) to Dutch finishing farms. Twelve farrowing farms, 11 finishing farms, six farrow-to finish farms, one rearing farm and one centre for artificial insemination were included. Screening of 310 pigs from these 31 farms showed 35 pigs (11%) to carry MRSA in their nares. On seven of the 31 (23%) investigated farms colonized pigs were found, including three finishing farms, three farrowing farms and one farrow-to-finish farm. The use of standard antimicrobial medication of the pigs seemed to be a risk factor for MRSA carriage. Screening of the pigs on six farms supplying pigs for the MRSA positive farms revealed that the pigs on all but one farm were MRSA positive. Genotyping revealed that all MRSA strains were non-typeable by PFGE using the *Sma*I restriction enzyme and had Multilocus Sequence Type (MLST) ST398. Different *spa*-types were found including t011, t108, t567, t899 and t1939, but the *spa*-types on epidemiologically related farms were identical indicating that MRSA are transmitted between farms through the purchase of colonized pigs. Two SCC*mec* types were found among the MRSA: type IV and type V. SCC*mec* type V was predominant. On two farms MRSA isolates with ST398, the same *spa*-type but with different SCC*mec* types (IV and V) were found, suggesting that different SCC*mec* elements have been inserted into MSSA with the same genotype. All MRSA strains were resistant to tetracycline, but additional resistances to erythromycin, lincomycin, kanamycin and gentamicin were also found. All MRSA isolates were negative for the exfoliative toxin genes (*eta* and *etb*), PVL toxin genes (*luk*F and *luk*S), toxic shock syndrome gene (*tst*-1), and the leukotoxin genes (*luk*E, *luk*D, *luk*M, *luk*F').

INTRODUCTION

In humans, methicillin-resistant *Staphylococcus aureus* (MRSA) are an important cause of hospital-acquired infections worldwide. MRSA is not confined to health care settings and during the last ten years community-acquired MRSA (CA-MRSA) has been reported increasingly as a cause of infection or colonization in healthy individuals, often in the absence of recognizable risk factors. In recent years, increasing numbers of reports have documented the occurrence of MRSA in animals, especially in dogs, cats and horses (Weese et al., 2000, van Duijkeren et al., 2004, Rankin et al., 2005). As in humans, MRSA can colonize skin, nasal and oral mucosae in healthy animals, or cause infections, especially wound infections (Moodley et al., 2006). Recently, Armand-Lefevre et al. (2005) identified pig farming as a risk factor for increased nasal *S. aureus* carriage. In the period 2004 to 2005, MRSA was cultured from three Dutch patients who had direct or indirect contact with pigs: an infant of a pig farmer, a son of a veterinarian and a pig farmer (Voss et al., 2005). These investigators also found six MRSA carriers among a group of 26 pig farmers. In 2006, screening of 540 fattening pigs at nine slaughterhouses all over The Netherlands showed that 39% of the pigs carried MRSA (de Neeling et al., 2007). Recently three other patients with infections caused by pig-associated MRSA were described (Huijsdens et al. 2006; Ekkelenkamp et al., 2006; Schneeberger et al., 2006). Interestingly, all of the MRSA isolated from the pigs and pig-associated human cases were non-typeable by PFGE using *Sma*I macrorestriction, and were of Multilocus Sequence Type (MLST) ST398, indicating clonal spread. Recently, Wulf et al. (2006) reported that MRSA carriage was 4.6 % among 152 Dutch veterinary students and veterinarians in contact with livestock. These findings have consequences for the Dutch search and destroy policy which has been very effective: in 2005, only 2% of the *Staphylococcus aureus* (*S. aureus*) isolates from Dutch hospitals were resistant to oxacillin (SWAB, 2006). In The Netherlands, contact with pigs is now recognized as a risk factor for MRSA carriage. To date, the reasons for the high prevalence of MRSA ST398 in Dutch finishing pigs are unknown. One possible explanation is that farrowing- and farrow-to-finish farms are also colonized and sell colonized pigs to the finishing farms.

The aims of the present study were (i) to determine the prevalence of MRSA in healthy pigs at different kinds of pig farms in order to identify possible sources of MRSA for finishing pigs (ii) to examine if the usage of antimicrobial drugs increases the risk of finding MRSA positive pigs on a farm (iii) to analyze the

porcine MRSA by Pulsed Field Gel Electrophoresis (PFGE), Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing, Multilocus Sequence Typing (MLST), *spa*-typing, the presence of genes encoding for toxins and susceptibility.

MATERIALS AND METHODS

Survey on the farms

In the period between August 2006 and November 2006, nasal swabs were collected from healthy pigs on 31 pig farms in The Netherlands: 12 farrowing farms, 11 finishing farms, six farrow-to finish-farms, one rearing farm and one centre for artificial insemination. All samples were convenience samples. Most farms (n= 25) belonged to the ambulatory clinic of the Veterinary Faculty and are located in Utrecht province. The other six farms are located in different provinces of The Netherlands. On each farm 10 randomly selected pigs were sampled and one nasal swab per pig, from both nares, was taken. On the centre for artificial insemination we sampled the boars, on the rearing farm the gilts, on the farrowing farms the weaned pigs, on the farrow-to-finish-farms the finishers and on the finishing farms the finishers. Each farmer was asked if the batch of pigs we sampled had been treated with antimicrobial drugs, what kind of antimicrobial drugs and at what moment. Only group treatments and not individual treatments of pigs were registered. In addition a swab was taken from the farmers on a voluntary basis, from the throat and from both nares, with subjects' informed consent. One of the farms, where the farmer was carrier of MRSA, was sampled a second time, after the pigs had been treated with tetracycline in order to cure a respiratory disease.

In the second part of our study, we sampled pigs at six farms supplying pigs for the MRSA-positive farms, in order to investigate whether farms obtain MRSA through the purchase of colonized pigs. One MRSA-positive farm from the first part of our study was a closed farm, so no supplying farm could be sampled. If the MRSA-positive farm was a farrowing farm or a farrow-to-finish-farm, samples were taken from 10 gilts on the supplying breeding farm; if the positive farm was a finishing farm, samples were taken from 10 weaned pigs on the supplying farrowing farm. The sampling was done as described above.

Bacterial culturing

The samples were incubated individually in MRSA broth, containing tryptic soy broth, 4% saline, 1% mannitol, phenol red (16 µg/ml), ceftizoxime (5 µg/ml) and aztreonam (50 µg/ml). The MRSA broth was incubated at 37°C for 48 h and then plated on sheep blood agar. The sheep blood agar was incubated at 37°C for 24 h. Suspected colonies were identified as *S. aureus* using standard techniques: colony morphology, Gram staining, catalase and coagulase and by Pasteurex Staph-plus (Bio-Rad). The identity of the isolates was confirmed by a PCR specific for the *nuc* (thermonuclease) and *mecA* (PBP2a) genes. As internal amplification control a fragment of the 16S rRNA genes was amplified. The primer sets used were 5'-GCGATTGATGGTGATACGGTT and 5'-AGCCAAGCCTTGA CGAACTAAAGC; 5'-GTTGTAGTTGTCGGGTTTGG and 5'-CTTCCACATAC CATCTTCTTTAAC; 5'-AGGCCCGGGAACGTATTCAC and 5'-GAGGAAGG TGGGGATGACGT, respectively (de Neeling et al., 1998; Braksted et al., 1992).

Antimicrobial susceptibilities were determined by an agar diffusion method using Neo-sensitabs discs (Rosco, Denmark). The antimicrobials tested were tetracycline (T), lincomycin (L), erythromycin (E), enrofloxacin (EN), trimethoprim/sulfamethoxazole (TS), gentamicin (G) and kanamycin (K). The breakpoints used were those recommended by the Dutch Committee on Guidelines for Susceptibility testing (CRG, 2000): for tetracycline, gentamicin, kanamycin and lincomycin zone diameters of ≥28 mm were regarded as susceptible and zone diameters of < 25 were regarded as resistant; for enrofloxacin zone diameters of ≥ 26 were regarded as susceptible and zone diameters < 23 were regarded as resistant; for erythromycin zone diameters ≥ 27 were regarded as susceptible and zone diameters < 23 as resistant; for trimethoprim/ sulfamethoxazole diameters of ≥28 were regarded as susceptible en zone diameters of < 23 as resistant.

Genotyping of the MRSA

The MRSA isolates were genotyped by PFGE using *Sma*I as restriction enzyme according to the Harmony protocol (Murchan et al., 2003), *spa*-typing (Harmsen et al., 2003) and Multilocus Sequence Typing (MLST) (Enright et al., 2000). Typing of the SCC*mec* was performed by PCR (Ito et al., 2001, Ito et al., 2004, Okuma et al., 2002).

Detection of toxin genes

The detection of the exfoliative toxin genes *eta* and *etb*, PVL toxin genes (*luk*F and *luk*S) (Lina et al., 1999), toxic shock syndrome gene (*tst*-1), and the leukotoxin genes (*luk*E and *luk*D) (Yamada et al., 2005) and *luk*M/F' (Jarraud et al., 2002) was performed by PCR. The primers used for the detection of *tst*-1 were ATTTTACCCCTGTTCCCTTATCATC and TAGGTGGTTTTTCAGTATTGT ATTCA, for *eta* and *etb* CATTTGGTGCAGGTGTTGATTT and TCCACGGATT TTTATTTTATTTATTAC and ATATTATTTTTACACCCGCTCAA and TTCCCCAA AGTGTCTCCAAAAGTA respectively.

RESULTS

On seven of the 31 (23%) investigated farms colonized pigs were found, including three finishing farms, three farrowing farms and one farrow-to-finish farm (Table 1). A total of 35 pigs (11 %) carried MRSA in their nares. The number of positive pigs ranged between 0 and 9 out of 10 pigs tested. Of the 10 farms on which antimicrobials were used as standard medication, six farms were MRSA positive. Of the 21 farms on which no standard medication with antimicrobials was used, only one farm was MRSA positive. On two of the seven MRSA-positive farms human MRSA carriers were found (3 colonized persons among 11 persons tested on 5 farms on which samples from humans were taken). On 18 of the 24 MRSA-negative farms, a total of 24 persons were tested and only one person was colonized with MRSA (on 6 MRSA-negative farms no samples from humans were taken). On one farm the farmer was colonized with MRSA, but the pigs were MRSA-negative. After the pigs had been treated with oxytetracycline for acute

respiratory disease, they were sampled a second time. The result of this second sampling was that 8 out of 10 pigs were MRSA positive.

On five of the six farms supplying pigs for the seven MRSA-positive farms the pigs were also MRSA positive. On five supplying farms human MRSA carriers were found (8 colonized persons among 11 persons tested). On the farm were all pigs were MRSA-negative, the only person tested was MRSA-positive. On one farm with MRSA-positive pigs, no human samples were taken (see Table 1). In total, colonized personnel was found on 8 out of 13 MRSA-positive farms investigated and only one of the 18 MRSA-negative farms investigated.

The MRSA strains had resistance patterns T, TE, TEL, TKG and TELKG. On three farms MRSA with two different resistance patterns (T and TGK; T and TEL) were found. All MRSA isolates were negative for the PVL leucocidin toxin genes (*luk*S and *luk*F), for the toxic shock syndrome gene (*tst*-1), the leukotoxin genes (*luk*E, *luk*D, *luk*M and *luk*F') and for exfoliative toxin A and B. All isolates were untypable by PFGE using *SmaI* digestion, and had ST398. Five related *spa*-types were found: t011, t108, t567, t899 and t1939 (Table 2). On the farms which were epidemiologically related, the same *spa*-types were found. The *spa*-types found in the pigs and the farmer on a farm were also identical. Two SCC*mec* types were found, SCC*mec* type IV and V. SCC*mec* type V predominated (Table 1). Remarkably, different SCC*mec* types (IV and V) were found among the MRSA isolates from the pigs on one farm in isolates with the same *spa*-type t011, but with different resistance patterns (TKG and T). On another farm the MRSA isolates cultured from the pigs had a SCC*mec* type differing from the SCC*mec* type of the isolates from the farmer, although the *spa*-types were identical.

Table 1. Data of the MRSA positive farms: farms number 1-7 are the MRSA positive farms investigated in the first screening of 31 farms. Farm A, B, C, D, E, F are the farms supplying the pigs for farms 1, 2, 4, 5, 6 and 7. Farm 3 is a closed farm.

No.	Kind of farm	Supplier of the pigs for the positive farms	Standard use of antimicrobial drugs/time	No. of positive pigs/ no. of pigs tested	No. of human carriers/ no. of persons tested	*Spa* type	SCC*mec* type	Resistance pattern*
1	farrow-finish		ampicillin doxycycline/at weaning	9/10	0/1	t011	IV V	TGK and T
2	finishing		trimethoprim-sulphonamides/ at 11 weeks	2/10	0/1	t899	V	T
3	farrowing	closed farm	colistin tulathromycin amoxicillin/	7/10	nt	t567	V	T
4	farrowing		colistin/at weaning	3/10	0/3	t108	V	TE
5	finishing		tylosin doxycycline/ therapeutic	3/10	1/2	t108	V	TEL
6	finishing		doxycycline / at weaning	2/10	nt	t011 t108	V	TEL
7	farrowing		amoxicillin/ at weaning	9/10	2/4	t567	V	T and TEL
A	multiplier	1	colistine	0/10	1/1	t011	IV	TELGK
B	farrowing	2	amoxicillin	8/10	nt	t899 t1939	V	TEL
C	multiplier	4	trimethoprim/ sulfonamides	2/10	1/1	t108	V	TE
D	farrowing	5	none	1/10	1/3	t108	V, IV	TEL
E	farrowing	6	tetracycline	8/10	1/1	t108	V	TEL
F	multiplier	7	tetracycline amoxicillin	4/10	4/5	t567	V	T and TEL

*T=tetracycline, E=erythromycin, L=lincomycin, G=gentamicin, K=kanamycin; nt= not tested

Table 2: *Spa* types and repeat succession of the MRSA isolates found in the present study.

Spa type	Tandem repeats	Number of farms with this type
t108	008-16---- 02-25---- 24-25	6
t011	008-16---- 02-25-34-24-25	3
t567	008 ------- 02-25---- 24-25	3
t899	007-16- 23-02---- 34------	2
t1939	007---- 23-02----34------	1

DISCUSSION

An important finding of our study was the occurrence of MRSA on different kinds of pig farms and their supplying farms. The *spa*-types of the MRSA isolates found on the farms and their respective supplying farms were identical. Porcine MRSA isolates with the rare *spa*-type t899, which has not been reported before in pigs, was found on a finishing farm and the farrowing farm supplying the piglets for the finishing farm. Together, these data indicate that finishing farms and farrowing farms may be colonized by MRSA through the purchase of colonized pigs from other farms. This knowledge is essential for choosing the right strategy for future programs aimed at controlling the spread of MRSA in pigs. However, importing colonized pigs is perhaps not the only way of propagation of MRSA, as one closed farm was also MRSA positive. MRSA may also be spread repeatedly between pigs on farms without an all-in all-out system; between colonized personnel/veterinarians and the pigs or between the contaminated environment and the pigs and therefore continual introduction is probably not necessary.

Another important finding of our study was that the use of standard antimicrobials for the pigs seems to be a risk factor for finding MRSA-positive pigs on a farm. Pig farms on which the pigs were treated with antimicrobials as group medication had a higher risk of being MRSA positive, whereas farms on which antimicrobials were used restrictively had a much lower chance of being MRSA positive. The MRSA isolates were resistant to beta-lactam antibiotics, tetracycline, aminoglycosides, lincosamides and macrolides and therefore the use of these antimicrobials may select for the pig-associated MRSA. Tetracyclines are extensively used as group medication in the Netherlands. Remarkably, all MRSA isolates were susceptible to trimethoprim/sulfonamides, although these drugs are

also used commonly in pigs. On one farm the pigs were MRSA positive after they had been treated with oxytetracycline. The farmer, however, was MRSA-positive during the first screening. Possibly the pigs were already colonized with MRSA before the treatment, but the level of colonization was below the limit of our detection method. Another cause might be that the respiratory disease predisposed the pigs to colonization.

The prevalence of MRSA-positive pig farms (23%) in our study was lower than the prevalence reported by de Neeling et al. (2007) (81%). An explanation for this might be that we included a large number of farms belonging to the ambulatory clinic of the Veterinary Faculty. On these farms the use of antimicrobial drugs is generally more restricted compared to other farms. Another reason might be that we sampled pigs on the farms, whereas in the other study (de Neeling et al., 2007) the pigs were sampled at slaughterhouses. The higher prevalence in the slaughterhouses may have been caused by cross contamination in the lairages, because the percentage of MRSA-positive pigs was significantly different between slaughterhouses (de Neeling et al., 2007). A third explanation might be differences between the sensitivity of the culture techniques used.

Colonized personnel was found more often at the MRSA-positive farms investigated indicating that farmers/personnel on MRSA-positive farms have a greater chance of being colonized with MRSA than farmers/personnel on MRSA-negative farms.

All MRSA isolates had MLST 398 and five different, but closely related *spa*-types were found suggesting clonal spread. However, on two farms MRSA isolates with the same *spa*-type but with different SCC*mec* types were found. This suggests that different SCC*mec* elements have been inserted into MSSA with the same genotype. The MRSA isolated from the pigs and pig-associated human cases are non-typeable by PFGE using *Sma*I macrorestriction, because they posses a restriction modification enzyme which methylates the *Sma*I-recognition sequence (Bens et al., 2006). We did not find Panton Valentine leucocidin toxin genes, the toxic shock syndrome gene, leukotoxin genes or exfoliative toxin A and B in the pig-associated MRSA isolates. However this MRSA could acquire one or more of these toxin genes because these genes can be present on mobile elements like plasmids and bacteriophages. If the pig-associated MRSA acquires one or more toxin genes this could increase its pathogenicity.

In conclusion, 23 % of the pig farms were MRSA positive and five out of six farms supplying pigs for the positive farms were also MRSA positive, indicating transmission within the production chain. The use of antimicrobial drugs as group medication was associated with finding MRSA colonized pigs on a farm.

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Chapter 7

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.

Table 1. Seven of *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.

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).

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^{o}C for 2 min and followed by hybridization of the target mixture to the prehybridized, prewarmed microarray slides. Hybridization was performed overnight at 42 ^{o}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.

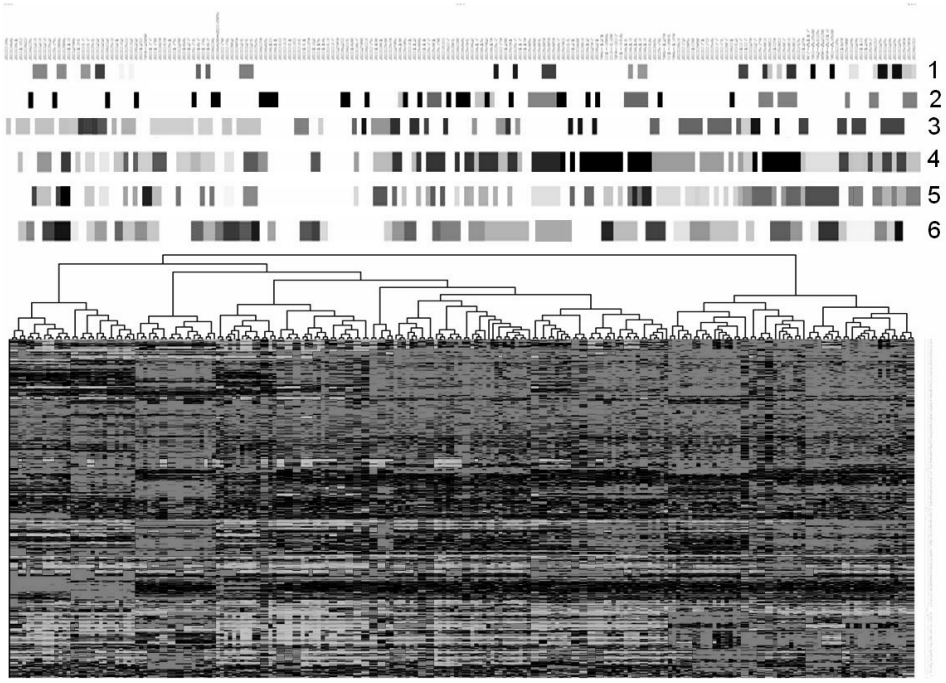

Figure 1. Two dimensional hierarchical clustering of the 164 *S. aureus* strains obtained from hybridization with 400 probes. Red and green spots represented the presence and divergence of genes (probes).The y-axis shows the 400 probes, while the isolates are on the x-axis. Clustering of the isolates 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 (see also the text for examples).

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	Number 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]

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 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.

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.

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.

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.

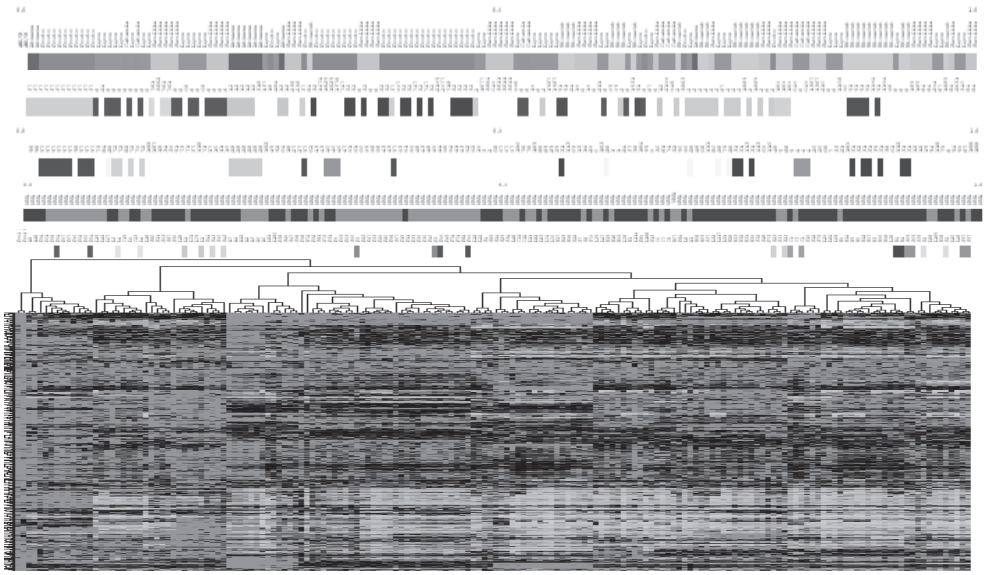

Figure 2.Two dimensional hierarchical clustering of 157 ICUs isolates using 400 probes. Shown from top to bottom clustering based on city of origin, *spa*-type, MLVA-type, MRSA/MSSA and duplicates.

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	Number 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 with 400 probes	61	0,982	[0.977 - 0.986]
Microarray with 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 using 400 probes	Microarray using 205 probes
MLVA	-			
Spa-typing	0,323	-		
Microarray using 400 probes	0,238	0,241	-	
Microarray using 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 (Fig. 3). The same analysis for the 157 ICU isolates resulted in 157 differentiating biomarkers, a 60% probe reduction (Fig. 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.

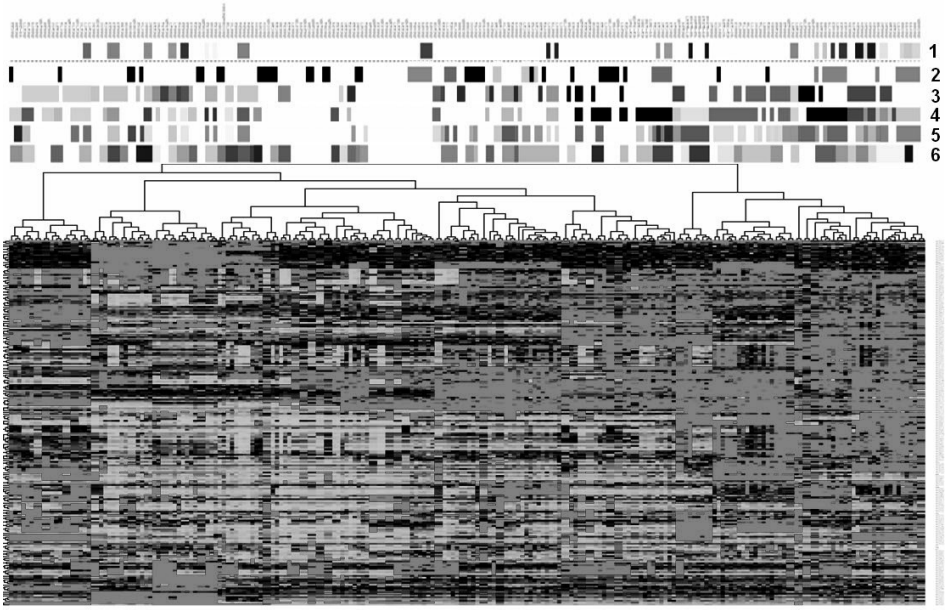

Figure 3. Two dimensional hierarchical clustering of 164 isolates with 205 probes after 45% of probes reduction (cut-off GD 6.6). Clustering of the isolates was determined based on (1) replicates; (2) epidemiological relatedness; (3) MLST types; (4) *spa*-types; (5) MLVA types; and microarray profile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

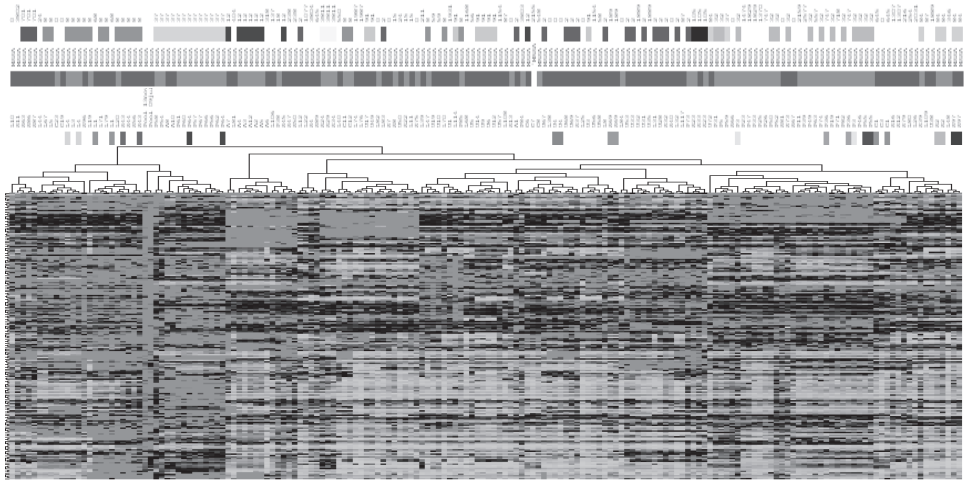

Figure 4. Two dimensional hierarchical clustering of 157 ICUs isolates using 205 probes. Shown from top to bottom clustering based on *spa*-type, MRSA/MSSA and duplicates.

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.
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 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	Number 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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Chapter 8

General discussion and summary

Since it emerged in the 1960s, MRSA has become a major problem with a wide range of both clinical and public health manifestations. In the early years, MRSA was confined to the hospital setting and effective control measures were known. It remained a significant cause of nosocomial morbidity and mortality. However, the nature of MRSA has started to change and now includes community settings. A worldwide increase of community-associated MRSA (CA-MRSA) outbreaks was observed in the mid 1990s. At present, humans are not the only and the most important reservoir for MRSA. MRSA has also emerged in animal reservoirs particularly in cattle and pigs (20,22). This MRSA can be transmitted to humans. Therefore, early recognition and detection of MRSA which may cause an outbreak is important for infection control.

A number of typing tools have been developed in order to have rapid and efficient means for MRSA detection. Various techniques have been employed to type *S. aureus*, from simple to very sophisticated methods, with different resolutions for solving a particular problem, i.e., in case of outbreaks or population studies. However, as described earlier in the Introduction, the currently available methods are not satisfactory. This is in particular true for outbreak detection. A rapid and accurate method is highly desirable. As response to this problem, we developed and evaluated novel typing systems for outbreak detection and population study purposes of *S. aureus* of both humans and animals. In addition, we investigated transmission of MRSA between pig farms and characterized virulence factors present in genotyped bovine mastitis isolates.

A typing method using variable number tandem repeats (VNTRs) for identification of *S. aureus* has been described before. This method employed a multiplex PCR of 5 genes, *sdr, clfA, clfB, ssp* and *spa* (18). However, it had a drawback since the interpretation was DNA fingerprint-based. In Chapter 2, we developed a new approach using the MLVA system by utilizing VNTR of *S. aureus*, termed staphylococcal interspersed repeat units (SIRUs) (11). Seven SIRUs (SIRU01, 05, 07, 13, 15, and 21) have been used for typing purposes of MRSA isolates within an endemic setting in an intensive care unit (ICU) in the United Kingdom at a very limited scale (10,11). In the beginning of the evaluation, we also added the *sspa* locus in our MLVA system, but later on *sspa* and SIRU15 were excluded because no variation in the repeat number was observed. In total 164 human clinical *S. aureus* isolates were used in this study, 100 randomly chosen isolates collected by the European Network for Antimicrobial Resistance and Epidemiology (ENARE) that have been genotyped by multilocus sequence typing

(MLST), 50 isolates represented outbreaks and epidemiologically unlinked strains collected by the Hygiene Department of the University Medical Centre Utrecht. Finally, 14 isolates from 4 patients that were taken during a 5 year period were included. Amplification of the 6 SIRUs resulted in a string of the number of repeat units. This was considered an allelic profile which we further designated as an MLVA type (MT). Unlike the first MLVA, we developed a character-based MLVA scheme. Thus, the interpretation uses an allelic profile rather than a fingerprint.

Evaluation of the performance of the typing system is based on several criteria including typeability, reproducibility, stability, discriminatory power, and epidemiologic concordance (17). The SIRUs of all isolates tested could be amplified. So, the MLVA scheme showed good typeability. Validation of this typing scheme demonstrated that it was able to distinguish outbreaks and incidental isolates which were originally identified by phage-typing or PFGE. We demonstrated that some isolates could be mistakenly clustered into an outbreak group when relationships were considered exclusively based on the phage type, whereas MLVA distinguished different MTs. Furthermore, we were able to demonstrate long term stability of this MLVA scheme. The method is highly reproducible. This is demonstrated by the fact that the number of repeats for SIRU21, which is the same repeat region of the spa gene used for *spa*-typing, always yielded the same number of repeats as obtained by DNA sequencing for spa-typing. Furthermore, this is the smallest repeat with only 24 bp.

Discriminatory power has been regarded as a key characteristic of typing systems (17) since it provides information about the discriminatory ability of typing methods. So, it is a measure how many different clusters of isolates can be detected. The novel MLVA scheme for typing human *S. aureus* had an excellent discriminatory power compared to MLST and spa-typing, and equal to pulsed-field gel electrophoresis (PFGE) based on Simpson's index diversity and a 95% confidence interval (13). Epidemiologic concordance was measured by Adjusted Rand's index and Wallace's coefficient that compared two sets of results obtained by different typing methods (5). The Adjusted Rand index and Wallace's coefficient indicated that MLVA was reasonably well predictive for both MLST and *spa*-type, but not vice versa. While MLVA and PFGE are poorly predictive for each other indicating that they are not based on the same genetic signal.

It should be noted that we observed variation of one or more loci within a group of outbreak isolates that had indistinguishable or closely related PFGE profiles.

Therefore, the MLVA typing criteria used for *Enterococcus faecium* were followed (19). These criteria are: Indistinguishable: isolates have an identical MLVA type; Closely related: isolates have MLVA types that differ only in one locus, also called single locus variant (SLV); Possibly related: isolates show MLVA types that differ in two loci, so-called double locus variants (DLV); Unrelated: isolates that have more than two loci different in their MLVA types. A decision to include or exclude an isolate from an outbreak, however, should always involve the epidemiological data of the isolates involved.

Since our MLVA scheme has shown great potential to be a fast and reliable typing method, we extended the use of this method to MSSA and MRSA of animal origin as described in Chapter 3 and Chapter 4.

In Chapter 3, we characterized 85 Dutch *S. aureus* from bovine clinical mastitis using the newly developed MLVA scheme, PFGE, *spa*-typing, and MLST. Thirty five isolates were collected by the Faculty of Veterinary Medicine Utrecht University that represented regional strains, 50 other isolates were from the collection of the Central Veterinary Institute, Lelystad, and represented national strains. Ninety percent of these isolates were obtained from different farms and when two isolates of a farm were included they were sampled at least one year apart, thus geographical bias could be excluded.

The MLVA scheme showed good typeability although SIRU07 and 13 were not always amplified, while SIRU05 was completely absent. SIRU21 is valuable in MLVA since it had the most variation in repeat units (RUs). We observed no particular difference between regionally and nationally obtained *S. aureus* from bovine mastitis. Simpson's index of diversity showed that PFGE had the highest discriminatory power followed by MLST, MLVA and *spa*-typing. Adjusted Rand index and Wallace's coefficient showed poor predictions of the MLVA to PFGE and MLST also indicating that MLVA was less discriminatory than MLST and PFGE. SIRU05 yielded no usable data and it may explain the low discriminatory power of MLVA. When the neighboring region of SIRU05 was analyzed in depth, we observed different genetic elements bordering the repeats which explained the lack of SIRU05 amplification. A fosfomycin resistance element that borders SIRU05 in human *S. aureus* isolates was absent in the bovine *S. aureus* isolates, while amplification of SIRU05 was based on primers that were developed for human *S. aureus* isolates. Furthermore, a single nucleotide mutation was detected upstream SIRU05 where a LysR encoding gene was located. LysR

proteins belong to a large family of regulatory proteins (12,24). This mutation introduced a stop-codon and inactivated translation of the substrate domain of the LysR protein. This may lead to different expression patterns in bovine *S. aureus* strains compared to the human strains.

The relationship of bovine mastitis *S. aureus* with the rest of the *S. aureus* population was performed by MLST. Bovine *S. aureus* belonged to different clusters than the human *S. aureus* population, with an exception of 4 isolates that belonged to CC97 that has previously been reported to harbor isolates obtained from humans (9). This finding supports the hypothesis of host-specificity of MRSA clones (1,14). This may reduce the chance transmission of human-derived MRSA to cattle, which may occur occasionally. Interestingly, we observed new MLST types (STs) among Dutch bovine mastitis which belonged to clonal complexes (CCs) that differed from those described elsewhere, which might suggest emergence of new MSSA clones. In addition, this study suggests that *spa*-typing was not a useful method for the typing of bovine mastitis, because of low variation among the spa-types observed. In addition, it was less discriminatory than MLST.

Studies in The Netherlands reported that more than 20% of the pig farmers and almost 40% of slaughterhouse pigs carried MRSA that belong to ST398 (7,23). Moreover, the ST398 MRSA has been proven to be transmitted not only to other animals such as cattle, but also to humans (8,15,20,21). The fact that large numbers of pigs are exported across national borders throughout the EU including millions of pigs from The Netherlands has raised concern about the MRSA problem in pig farms. Since our MLVA scheme was also applicable for *S. aureus* from bovine mastitis, we extended and evaluated the use of this method to pig-associated MRSA isolates as reported in Chapter 4.

In Chapter 4 we describe the results of typing 50 Dutch pig-associated MRSA ST398 isolates collected by the Faculty of Veterinary Medicine, Utrecht University, with the MLVA scheme. Only *spa*-typing was performed, since pig-related *S. aureus* belong to a single ST (398) and have been recognized to be non-typeable by PFGE using *Sma*I (3). MLVA displayed a higher discriminatory power than *spa*-typing and very well predicted the *spa*-types. Almost all isolates belong to the *spa*-types that are commonly associated with ST398.

The MLVA showed that all SIRUs could be amplified except SIRU05; a similar phenomenon as described for the bovine mastitis isolates (Chapter 3). Thus, it is

likely that the fosfomycin resistance element was also absent in the pig-associated MRSA. Interestingly, we detected a double fragment in amplification of SIRU13 in all isolates. This was also observed previously for two clinical human pig-related *S. aureus* isolates (Chapter 2). Investigation of the region neighboring SIRU13 showed duplication upstream the "original" SIRU13 of a 162 bp fragment. SIRU13 is located upstream of the *hmrB* gene, a gene that can convert heterogeneously expressed methicillin resistance to homogenously expressed methicillin resistance (16). In addition, the SIRU13 region is highly variable among different isolates. From this finding, we hypothesize that SIRU13 may be involved in regulation of methicillin resistance.

In conclusion, the MLVA scheme used performs very well for human *S. aureus* isolates and is suitable for typing of *S. aureus* isolates from bovine mastitis and for pig-related MRSA. However, because the amplification of some loci and in particular SIRU05 in the latter two groups is poor, the replacement of SIRU05 by another locus or the addition of another locus should be investigated. Ideally, this new locus should also be used for human *S. aureus* isolates to keep the MLVA scheme as universal as possible.

In Chapter 5, virulence factor encoding genes in bovine mastitis *S. aureus* were investigated to get insight into the genetic determinants that underlie mastitis. A set of virulence genes including 19 toxin genes, 12 adhesin genes, 3 immune evasion genes, and 6 other genes were examined by PCR amplification for their presence in 76 Dutch bovine mastitis *S. aureus* isolates that had been extensively genotyped by PFGE, MLST, *spa*-typing, and MLVA (see Chapter 3). Based on the absence and presence of genes, virulence factor gene profiles were generated. Five major virulence factor profiles were obtained. We compared the 5 virulence profiles with the clustering based on the other four typing methods, and we observed general agreement with an exception of 2 clusters due to the presence of a bovine-specific *S. aureus* pathogenicity island (SaPIbov). This suggests independent mobilization of SaPIbov. A minimum set of genes was distilled from the resulting virulence factor gene profiles. Six genes including the gene for β-hemolysin (*hlb*), elastin-binding protein (*ebps*), serine protease (*sspA*), cysteine protease (*sspB*), fibronectin-binding protein A (*fnbA*), and coagulase (*coa*) seem to be necessary for the mastitis phenotype in bovines. However, this does not exclude the possibility of other genes to contribute to the severity of disease. Another possibility is that different gene profiles explain bovine mastitis.

Chapter 6 described the occurrence of MRSA on different kinds of pig farms and their supplying farms. Ten randomly selected pigs from 31 pig farms including 12 narrowing farms, 11 finishing farms, 6 farrow-to-finish-farms, 1 rearing farms and 1 centre for artificial insemination were sampled for *S. aureus*. Twenty-five participating farms were located in Utrecht province and belonged to the ambulatory clinic of Faculty of Veterinary Medicine, Utrecht University. The other six farms were located in different provinces in The Netherlands. The use of antimicrobial drugs was noted. Isolates were also taken from the farmers on a voluntary basis. In addition, 6 supplying farms were included to investigate whether farms obtained MRSA through the purchase of colonized pigs, but 1 farm could not be sampled since it was a closed farm.

Antimicrobial susceptibilities were determined and genotyping was performed with PFGE using *Sma*I, MLST, spa-typing and SCCmec typing. Identical *spa*-types were observed on the farms and their respective supplying farms, which indicate colonization of MRSA through the purchase of MRSA carrying pigs. However, a closed farm was also MRSA–positive, which suggested that spread of MRSA was not only due to importing colonized pigs.

The MRSA strains had resistance patterns T, TE, TEL, TKG and TELKG (T: tetracycline; E: erythromycin; L: lincomycin; K: kanamycin; G: gentamicin). Three farms showed 2 different resistance patterns (T and TGK; T and TEL). This is in agreement with the use of oxytetracycline for the treatment of pigs. So, oxytetracycline use seems to be a risk factor for finding MRSA-positive pigs.

This study observed a lower prevalence of MRSA-positive pig farms than a previous study (7) which might due to the fact that a large number of farms have been participating in the latter study. It should be noted, that the use of antimicrobial drugs in the farms in our study was generally more restricted. Other explanations could be that the study was restricted to pigs from farms and not from slaughterhouses or it might be due to a difference in the sensitivity of the culture methods used. We observed a high risk of being colonized with MRSA for farmers that work at the MRSA-positive farms. In addition, on two farms MRSA isolates with identical *spa*-type but different SCC*mec* types were observed. This indicates insertion of different SCC*mec* elements into MSSA with the same genotype. The pigs and pigs-associated MRSA could not be digested by *Sma*I due to the presence of a restriction modification enzyme which methylates the *Sma*I- recognition site (3). Furthermore, no toxin genes were detected. We

assumed that acquisition of one or more toxin genes to the pig-associated MRSA could increase its pathogenicity. In addition, all isolates were MRSA ST 398 and belong to five closely related spa-types suggesting clonal spread. At the moment of the study our MLVA typing scheme was not available, but it could have been valuable for quickly analyzing the spread of MRSA between farms. Also the microarray used for genotyping would be of great use for studying these types of problems, especially when probes are added for specific virulence factors and resistance genes (see Chapter 7).

The use of DNA microarray technology for identification of *S. aureus* on outbreak and population levels was described in Chapter 7. A total 164 clinical human *S. aureus* isolates were subjected to microarray using 400 probes. These isolates have been extensively genotyped by MLST, spa-typing and MLVA (Chapter 2). In addition, 157 genotyped *S. aureus* isolates from 6 European ICUs were included (4). All isolates could be hybridized and normalization of core genome probes showed that some isolates yielded low signals, but they were not excluded from analysis. Microarray profiles (APs) were defined based on the combination of presence and divergence of the probes, while clustering of APs was determined by a genetic distance (GD) cut-off obtained from replicates. Two GD cut-offs were applied for two different purposes. The first for identification of outbreak isolates and the second for population structure analysis.

From Simpson's index of diversity, Adjusted Rand index and Wallace's coefficient, we observed that microarray had a good typeability, reproducibility and stable. The microarray displayed a discriminatory ability similar to MLVA, while epidemiologic concordance analysis showed that the microarray data could reasonably predict MLST and *spa*-typing results in both groups of isolates. But most importantly, the microarray could classify outbreak isolates correctly, although a few isolates were not assigned in accordance with their assignment by the Department of Infection Control and Infection Prevention of the University Medical Centre Utrecht, Utrecht, The Netherlands. This might explained by a (minor) variation in the accessory genes which were "unstable" since they were more prone to gain and loss of genetic information between closely related strains (2,6). A similar result was obtained for the MLVA scheme (Chapter 2). Furthermore, the original assignment to outbreak or unrelated isolate was used as "gold standard", but these assignments might as well have been incorrect.

The number of significant probes was distilled by means of a statistical technique termed Significance Analysis for Microarray (SAM). Two hundred five significant probes were required to obtain similar results for discriminatory power and epidemiologic concordance to those obtained with 400 probes in both groups of isolates.

The same set of 400 probes with a different, higher, cut-off was applied for the group of 164 isolates to evaluate the microarray's potential for population studies. MLST was used as reference method since it is most widely used to study the population of *S. aureus* and has been shown to clearly discriminate distinct lineages. We observed that microarray could reasonably predict the MLST results and we conclude that the microarray can be used for population study.

Our results show a major advantage of microarray-based compared to other typing methods. It allows typing at both the hospital outbreak and population structure level using the same dataset. However, a number of issues need to be improved such as the genetic distance used as a cut-off for the assignment of isolates should be defined better, and probes that give irreproducible results should be eliminated. Addition of probes for specific virulence factors, e.g., as shown for bovine mastitis and resistance elements may further enhance the values of a small microarray for typing *S. aureus* isolates.

In summary, the novel MLVA scheme we developed seems to be a good tool for epidemiological tracing of MSSA and MRSA from human and animal origin. The method is fast, cheap, highly discriminatory, reproducible, stable and portable, which can be widely implemented in the institutions without access for DNA sequencing. The usefulness of the scheme would be greatly enhanced when a library with a MLVA web-based database for international comparison is set up. A microarray with a limited number of probes can be a useful typing method for *S. aureus* on the level of outbreak identification and population study by using different cut-offs. The quality of the microarray should be improved by a well-defined genetic distance cut-off, removal of unstable probes, i.e., probes that can not be called present or divergent in an unambiguous way, and inclusion of probes for specific virulence factors or resistance genes which may further enhance the utility of the microarray. Examples are the virulence genes detected in *S. aureus* that cause mastitis. Certain genes appear to be required for the pathogenesis of mastitis in bovines, although an alternative explanation is that different gene profiles lead to mastitis. However, further study is needed in order

to confirm this finding. Our study on the transmission of MRSA between pig farms, that showed that transmission of pig-associated MRSA within the production chain can occur, would have profited from the typing schemes we proposed if they were available at the time of the study.

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Chapter 9

Nederlandse samenvatting

Door een microscoop zien *Staphylococcus aureus* bacteriën er uit als geel-oranje gekleurde druiven clusters. De bacterie komt wijd verspreid voor en kan bij gezonde mensen op diverse plaatsen gevonden worden zoals neus en perineum. Twintig procent van de mensen is permanent drager, 60% is zo nu en dan drager en bij 20% wordt de bacterie niet of nauwelijks aangetroffen. *S. aureus* kan een scala aan ziektebeelden veroorzaken, waarbij de meeste infecties veroorzaakt worden door stammen waarvan de patiënt al drager was.

S. aureus is een groot probleem voor ziekenhuizen en de volksgezondheid. In de jaren na de opkomst van meticilline resistente *S. aureus* (MRSA) kwam deze alleen voor in de gezondheidszorg, waar MRSA verantwoordelijk werd voor veel morbiditeit en mortaliteit. Deze situatie heeft ruim 30 jaar geduurd, maar midden jaren negentig kwam MRSA steeds vaker voor buiten het ziekenhuis bij mensen zonder band met de gezondheidzorg. MRSA blijft evolueren en vormt een bedreiging door uitbraken en wereldwijde verspreiding. Op dit moment is de mens niet langer het grootste en belangrijkste reservoir. MRSA is enige jaren geleden opgedoken in de veeteelt en in het bijzonder bij varkens en kalveren. Deze MRSA kan ook de mens koloniseren. Het is daarom belangrijk om dat MRSA die een uitbraak kan geven snel herkend wordt om infectie preventie maatregelen te kunnen nemen.

Om MRSA te kunnen typeren zijn diverse technieken ontwikkeld. Sommige van deze technieken zijn beter geschikt voor het typeren op populatie niveau terwijl andere meer geschikt zijn voor het aantonen van uitbraken. Maar vooral de technieken voor het aantonen van uitbraken zijn niet ideaal.

Wij ontwikkelden een typeringsschema op basis van Multi-Locus Variable number of tandem repeat (VNTR) Analysis (MLVA). Dit schema werd geëvalueerd voor *S. aureus* isolaten afkomstig van mensen, mastitis bij koeien en isolaten die gerelateerd zijn met MRSA bij varkens (**Hoofdstuk 2-4**). Dit schema maakt gebruik van een eenvoudige DNA amplificatie van 6 VNTRs die Staphylococcal Interspersed Repeat Units (SIRUs) genoemd worden. De gebruikte VNTRs zijn SIRU01, -05, -07, -13 en -21. Na amplificatie wordt het aantal repeat units berekend. Dit resulteert in een cijfer reeks van repeat units. Deze cijfer reeks wordt als een allel profiel gezien en elk profiel is een MLVA type (MT). Deze MLVA verschilt van andere MLVA schema's door het gebruik van de berekening van het aantal repeat units in plaats van bandenpatronen (fingerprints). Het MLVA schema werd gevalideerd en vergeleken met beschikbare

typeertechnieken zoals Pulsed-Field Gel Electrophoresis (PFGE), Multi-Locus Sequence Typing (MLST) en staphylococcal Protein A typering (*spa*-typering).

Evaluatie van de MLVA was gebaseerd op typeerbaarheid, reproduceerbaarheid, stabiliteit, discriminerend vermogen en epidemiologische concordantie. Discriminerend vermogen is een hoofdkenmerk van typeringsmethoden. Het discriminerend vermogen wordt berekend met de Simpson's index of diversity. De concordantie tussen methoden wordt bepaald aan de hand van de Adjusted Rand index en Wallace's coefficient.

In **Hoofdstuk 2** beschrijven het testen van het schema op 100 willekeurig gekozen isolaten die door het European Network for Antimicrobial Resistance and Epidemiology (ENARE) verzameld waren en waarvan het Sequence Type (ST) door MLST bepaald was; 50 isolaten die in het verleden als epidemiologisch niet verwant of als uitbraak isolaat getypeerd zijn door de afdeling Ziekenhuishygiëne met behulp van faagtyperings- of PFGE gegevens; 14 isolaten verzameld over een periode van 5 jaar die afkomstig zijn van 4 patiënten. De methode had een uitstekende typeerbaarheid want alle SIRUs van alle isolaten konden worden geamplificeerd. Validatie van de methode met de isolaten van de afdeling Ziekenhuishygiëne toonde aan dat vrijwel alle isolaten op dezelfde wijze geclassificeerd werden. Aan de hand van de MLVA gegevens konden we ook laten zien dat op basis van faagtypering isolaten ten onrechte tot een uitbraak gerekend konden worden. De repeat aantallen blijken ook op de lange termijn voldoende stabiel voor uitbraak detectie. De methode is zeer reproduceerbaar. Dit werd aan getoond met behulp van SIRU21. SIRU21 is het *spa*-repeat gebied dat gebruikt wordt voor *spa*-typering. *Spa*-typering, dat gebruikt maakt van DNA sequentie bepaling, leverde altijd hetzelfde repeats als MLVA. SIRU21 is met een repeat van 24 baseparen ook de kleinste repeat unit. Het discriminerend vermogen van het MLVA schema was beter dan dat voor MLST en *spa*-typering en vergelijkbaar met dat van PFGE. Op basis van de Adjusted Rand index en Wallace's coefficient kon worden vastgesteld dat de MLVA redelijk voorspellend was voor zowel MLST als *spa*-typering, maar niet omgekeerd. MLVA en PFGE zijn niet voorspellend voor elkaar wat aangeeft dat deze methoden verschillende genetische eigenschappen bepalen. Wel namen we enige variatie waar in één of meer loci van isolaten die in de PFGE niet te onderscheiden of nauw verwant waren. Daarom werden eerder voor enteroccocen voorgestelde indelingscriteria gebruikt: Niet te onderscheiden: de isolaten hebben een identiek MT; Nauw verwant: de isolaten verschillen in één locus (single-locus variant of SLV);

Mogelijk verwant: isolaten verschillen in twee loci (double-locus variant of DLV); Niet verwant: de isolaten verschillen in meer dan twee loci. Echter een isolaat behoord pas tot een uitbraak als er ook epidemiologische data zijn die de verwantschap ondersteunen.

Omdat het schema er zeer veel belovend uitzag werden ook MSSA isolaten van mastitis bij koeien en MRSA afkomstig van varkens getest. Dit is beschreven in respectievelijk **Hoofstuk 3 en 4**.

In **Hoofdstuk 3** wordt de typering van 85 isolaten afkomstig van mastitis bij koeien met MLVA, PFGE, MLST en spa-typering beschreven. Vijfendertig isolaten waren verzameld door de Veterinaire Faculteit van de Universiteit en kwamen uit de regio Utrecht en 50 isolaten waren afkomstig uit de collectie uit het Centraal Veterinair Instituut en werden in heel Nederland verzameld. Negentig procent van de isolaten kwam van verschillende bedrijven en als er 2 isolaten van hetzelfde bedrijf gebruikt werden, waren deze met een tussentijd van tenminste een jaar verzameld. Er was dus geen sprake van overrepresentatie van een bepaald bedrijf.

Het onderzoek toonde aan dat het MLVA schema voor deze isolaten een behoorlijke typeerbaarheid had. SIRU05 werd niet geamplificeerd en in sommige gevallen konden ook SIRU07 en SIRU13 niet geamplificeerd worden. De meeste variabiliteit werd waargenomen in SIRU21 waardoor deze het meest waardevol was in het MLVA schema. Er waren geen bijzondere verschillen in de uitslagen van de typeringen voor de regionaal en nationaal verkregen isolaten. Het discriminerend vermogen was het grootst voor PFGE gevolgd door MLST, MLVA en spa-typering. De Adjusted Rand index en de Wallace's coefficient lieten zien dat MLVA slecht de uitkomst van PFGE en MLST voorspelt, wat in overeenstemming is met het feit dat MLVA minder discriminerend is dan deze technieken. Het relatief lage discriminerend vermogen kan in belangrijke mate verklaard doordat SIRU05 niet geamplificeerd werd.

Een nadere analyse van het gebied rondom SIRU05 liet zien dat de repeats door verschillende genetische elementen begrensd wordt in isolaten van afkomstig van mastitis en mensen. Een fosfomycine coderend genetisch element dat aanwezig is in isolaten van humane herkomst is afwezig in de mastitis isolaten. Dit verschil verklaart de afwezigheid van amplificatie in de isolaten afkomstig van mastitis. Tevens werd een puntmutatie gevonden in het gen voor LysR dat upstream van

SIRU05 ligt. LysR eiwitten behoren tot een grote familie van regulatoire eiwitten. Deze mutatie introduceert een stop-codon tussen het deel van het gen dat codeert voor het DNA bindend domein en het domein dat het substraat herkent. Hierdoor kan slechts een incompleet eiwit gevormd worden. Als gevolg hiervan kunnen onder nog onbekende condities verschillen in expressie van meerdere genen ontstaan tussen isolaten van de mens of mastitis bij koeien.

De gegevens van de MLST zijn gebruikt om de positie van mastitis isolaten in de populatie structuur van *S. aureus* te bepalen. De mastitis isolaten behoren tot andere clusters dan isolaten van menselijke oorsprong. Hier waren maar vier uitzonderingen op. Deze isolaten behoorden tot Clonal Complex 97 (CC97) waarin zich isolaten bevinden van menselijke oorsprong. Deze bevinding ondersteunt de hypothese dat *S. aureus* kloons gastheer specificiteit vertonen. Dit kan de kans op succesvolle overdracht van MRSA van de mens verminderen, hoewel dit nu en dan wel gebeurt. Opmerkelijk genoeg werden ook nieuwe STs gevonden bij isolaten van mastitis bij koeien die niet behoorden to CCs die door anderen beschreven zijn. Verder suggereert deze studie dat *spa*-typering niet erg bruikbaar is door de geringe variabiliteit. Het was bovendien minder discriminerend dan MLST.

In **Hoofstuk 4** worden de resultaten beschreven van MLVA en *spa*-typering op ST398 isolaten die door de Faculteit Geneeskunde verzameld zijn. PFGE is op deze isolaten niet uitgevoerd omdat het DNA niet door *Sma*I geknipt wordt.

Alle SIRUs, behalve SIRU05, konden worden geamplificeerd. SIRU05 wordt waarschijnlijk om dezelfde reden niet geamplificeerd als bij de mastitis isolaten (**Hoofdstuk 4**). MLVA had een beter discriminerend vermogen dan *spa*-typering en MLVA was voorspellend voor het *spa*-type. Opmerkelijk was dat amplificatie van SIRU13 in alle gevallen leidde tot de vorming van twee producten. Dit fenomeen hadden we al eerder waargenomen voor twee menselijke klinische isolaten die varkens-gerelateerd waren en ook tot ST398 behoorden (**Hoofdstuk 2**). Mede op basis van DNA sequentie gegevens werd de bepaling van het aantal repeat units gebaseerd op het kleinste product. Onderzoek van de DNA sequenties die SIRU13 flankeren liet een duplicatie van 162 baseparen upstream van SIRU13 zien. Een vergelijking van het SIRU13 locus laat zien dat gebied bijzonder variabel is en in staat is secundaire structuren te vormen. Downstream van dit locus ligt *hmrB*, een gen waarvan aangetoond is dat het betrokken kan zijn bij de conversie van heterogene resistentie voor meticilline naar homogene

resistentie. Onze hypothese is dat SIRU13 betrokken is bij de regulatie van *hmrB* expressie en daarmee de overgang van heterogene naar homogene expressie.

In **Hoofdstuk 5** zijn de virulentie gen profielen van de isolaten afkomstig uit mastitis bij koeien, die in zolas in **Hoofdstuk 3** beschreven gegenotypeerd waren, bepaald met als doel een beter inzicht te krijgen in welke virulentie factoren noodzakelijk zijn voor het oplopen van mastitis. De aan- of afwezigheid van de genen voor 19 toxines, 12 adhesie eiwitten, 3 immuunsysteem remmende eiwitten en 6 andere virulentie factoren werd vastgesteld met behulp van PCR. De meeste isolaten konden gegroepeerd worden in 5 virulentie gen profielen. Deze 5 groepen waren in overeenstemming met de indeling op basis van genotypering. Er waren 2 uitzonderingen ten gevolge van het pathogenicity island SaPIbov. Deze bevinding suggereert dat SaPIbov tenminste twee maal overgedragen is naar deze stammen. Tenminste 6 genen (voor hemolysin ß (*hlb*), elastine bindend eiwit (*ebp*), serine protease (*sspA*), cysteïne protease (*sspB*), fibronectine bindend eiwit A (*fnbA*) en coagulase (*coa*)) lijken noodzakelijk voor het ontstaan van mastitis bij koeien. Dit sluit niet uit dat er andere genen bij betrokken zijn. Andere genen kunnen bovendien bijdragen aan de ernst van de infectie. Een andere mogelijkheid is dat meerdere virulentie gen combinaties aanleiding geven tot mastitis bij koeien.

Hoofdstuk 6 beschrijft de het onderzoek naar de overdracht van MRSA tussen varkenshouderijen in Nederland. Dit onderzoek betrof 12 fokbedrijf, 11 vleesvarkensbedrijven, 6 vermeerderingsbedrijven, 1 opfokbedrijven en een centrum voor kunstmatige inseminatie. Vijfentwintig van deze varkenshouderijen behoorden bij de ambulante kliniek van de Faculteit Geneeskunde van de Universiteit Utrecht. De overige varkenshouderijen bevonden zich in verschillende Nederlandse provincies. Het antibioticum gebruik werd genoteerd. Op vrijwillige basis werden ook isolaten van de varkenshouders verzameld. Verder werden 6 toeleveringsbedrijven geïncludeerd om vast te stellen of MRSA werd binnengebracht door de aankoop van varkens, maar één varkenshouderij kon niet bemonsterd worden omdat dit een gesloten varkenshouderij was. *Spa*-typering liet zien dat dezelfde types gevonden werden op varkenshouderijen en aanleveringsbedrijven. Dit suggereert dat tenminste sommige bedrijven MRSA verwerven door de aankoop van gekoloniseerde varkens. Echter een gesloten bedrijf was ook MRSA positief, dus aankoop van gekoloniseerde varkens lijkt niet de enige route. De varkenshouders bleken een grote kans te hebben om gekoloniseerd te raken. Ten slotte bleek dat er op twee bedrijven isolaten met

een identiek *spa*-type waren, maar met een verschillend SCC*mec* type. Dit duidt op de insertie van verschillende SCC*mec* elementen in MSSA met dezelfde genetische achtergrond.

De MRSA stammen hadden de antibioticum resistentie profielen T, TE, TEL, TKG en TELKG (T:tetracycline; E: erythromycine; L: lincosamine; K: kanamycine; G: gentamicine). Op 3 varkenshouderijen kwamen twee patronen voor (T en TGK; T en TEL). De resistentie voor tetracycline is overeenstemming met het gebruik van oxytetracycline in de varkenshouderij. Het gebruik is vrijwel zeker een risicofactor voor het verwerven van MRSA bij varkens. Er konden geen genen voor belangrijke toxine genen aangetoond worden. Alle isolaten behoorden tot ST398 en 5 verwante *spa*-types. Helaas was het MLVA typeerschema nog niet beschikbaar op het moment van deze studie.

Hoofdstuk 7 beschrijft het onderzoek om een microarray met een beperkt aantal probes te gebruiken voor het typeren van isolaten op zowel uitbraak als populatie niveau. Er werden 164 isolaten getest op een microarray met 400 probes. Deze isolaten waren al uitgebreid gegenotypeerd (**Hoofdstuk 2**). Verder werden 157 gegenotypeerde isolaten die verzameld zijn op 6 verschillende Europese intensive care units getest. Van alle isolaten werd een hybridisatie patroon verkregen waarbij voor elk gen werd vastgesteld werd of het aanwezig was, divergent was of dat de kwaliteit van het signaal onvoldoende was. Hoewel sommige patronen niet optimaal waren, werden zij niet uit de analyse verwijderd. Verkregen patronen worden microarray profielen (AP) genoemd. Clustering van de APs was gebaseerd op genetische afstand (GD) gebaseerd op duplo en triplo experimenten met een beperkt aantal isolaten. Er werden twee GDs bepaald: één voor het vaststellen van uitbraken en één voor populatie studies.

De microarray heeft een goede typeerbaarheid, reproduceerbaarheid en stabiliteit. Het discriminerend vermogen is vergelijkbaar met dat van MLVA. Concordantie gegevens toonden aan dat de microarray data redelijk voorspellend waren voor MLST en *spa*-typering in beide isolaat groepen. Maar belangrijker was dat op een enkele uitzondering na de isolaten die tot uitbraken behoorden goed geclassificeerd werden. Dit kan mogelijk verklaard worden door (geringe) variatie van de accessoire genen. Deze genen liggen bijna zonder uitzondering op mobiele genetische elementen. Het is echter ook niet uit te sluiten dat de clustering in uitbraken door de afdeling Ziekenhuishygiëne niet geheel correct

was. De groepering kwam ook redelijk overeen met de groepering op basis van MLST die veel voor populatie studies gebruikt wordt.

Met behulp van Significance Analysis voor Microarrays werd het aantal probes gereduceerd tot 205 probes zonder verlies van discriminerend vermogen en een vergelijkbare epidemiologische concordantie als verkregen met de 400 probes. Echter de cut-off definitie op basis van de GD moet verbeterd worden en probes die slecht reproduceerbare resultaten geven moeten verwijderd worden. Het toevoegen van specifieke probes bijvoorbeeld voor toxine of antibioticum resistentie genen kan de waarde van de microarray verder verhogen.

Samenvattend kan gezegd worden dat het nieuwe MLVA schema zeer bruikbaar is voor het typeren van MSSA en MRSA van zowel humane als dierlijke oorsprong. De methode is snel, goedkoop, zeer discriminerend, reproduceerbaar, stabiel en draagbaar, waardoor het makkelijk toepasbaar is ook voor instituten zonder uitgebreide moleculaire infrastructuur. De waarde van het schema zou nog vergroot worden door het opzetten van een website vergelijkbaar met die voor MLST of *spa*-typering, zodat data (inter)nationaal gestandaardiseerd en vergeleken kunnen worden. Microarrays met een beperkt aantal probes kunnen een waardevolle typeringsmethode worden. Een belangrijk voordeel is dat met verschillende clusteringen dezelfde data gebruikt kunnen worden voor het vaststellen van uitbraken en studies op populatie niveau. De kwaliteit van de microarray moet nog wel verder verbeterd worden en er is ook ruimte om de bruikbaarheid verder te vergroten. Verder blijken bepaalde genen noodzakelijk te zijn voor het ontstaan van mastitis bij koeien, hoewel een alternatieve verklaring is dat verschillende combinaties van virulentie factoren leiden tot dit ziektebeeld. De studie van MRSA in de varkenshouderij toonde aan dat er verspreiding plaatsvindt in de productieketen. De beschikbaarheid van het MLVA schema zou een belangrijk voordeel voor deze studie betekend hebben.

Chapter 10

Ringkasan dalam Bahasa Indonesia

Staphylococcus aureus (*S. aureus*) adalah bakteri yang tampak berwarna kuning hingga jingga, berbentuk kokus dan tersusun seperti buah anggur pada pengamatan dengan menggunakan mikroskop. Bakteri ini dapat ditemukan dimana-mana, dan pada manusia yang sehat biasa ditemukan di kulit, hidung, dan tenggorokan. Dua puluh persen dari populasi manusia membawa *S. aureus* secara terus menerus, 60% kadang-kadang, dan 20 % hampir tidak pernah membawa bakteri ini. Berbagai macam infeksi disebabkan oleh *S. aureus* dengan sebagian besar infeksi tanpa menunjukkan gejala penyakit.

S. aureus telah menjadi masalah utama di banyak rumah sakit dan juga bagi kesehatan masyarakat secara luas. Pada tahun-tahun awal ditemukannya *S. aureus* yang kebal terhadap antibiotika methicillin (methicillin-resistant *Staphylococcus aureus* atau MRSA), bakteri jenis ini masih terbatas sebagai masalah diruang lingkup rumah sakit dan dikenal sebagai mikroorganisme penyebab utama morbiditas dan mortalitas pada infeksi nosokomial. Keadaan ini berlangsung selama kurang lebih 30 tahun. Pada pertengahan tahun 90-an, MRSA menjadi lebih sering ditemukan diluar ruang lingkup rumah sakit, terutama pada orang yang tidak mempunyai riwayat kunjungan ke rumah sakit sebelumnya. Sampai saat ini MRSA terus berevolusi dan meluas melalui perjangkitan dan penyebaran secara pandemik.

Pada masa sekarang, manusia tidak lagi menjadi tempat hidup utama *S. aureus.* Habitat alami MRSA mulai bergeser ke hewan, misalnya pada binatang ternak seperti sapi dan babi. Lebih lanjut lagi, bakteri ini dapat ditransmisikan ke manusia. Oleh karena itu, pengenalan dan pendeteksian dini MRSA yang dapat mengakibatkan perjangkitan, sangatlah penting untuk mengontrol infeksi.

Beberapa teknik untuk mengidentifikasi *S. aureus* telah dikembangkan dengan derajat resolusi yang berbeda, baik untuk pendeteksian terjadinya perjangkitan ataupun untuk studi populasi. Walaupun demikian, metode yang tersedia pada saat ini dirasakan kurang memuaskan.

Kami mengembangkan dan mengevaluasi suatu metode baru dengan menggunakan beberapa lokus penanda genetik molekuler yang disebut “variable number tandem repeat” atau VNTR. VNTR ini tersusun atas pengulangan basa, yang jumlahnya bervariasi tiap lokusnya. Adapun metodenya dikenal sebagai “multiple locus VNTR analysis” atau MLVA, yang penggunaannya ditujukan untuk mendeteksi perjangkitan dan studi populasi dari bakteri *S. aureus* yang berasal

dari manusia dan hewan ternak,,seperti yang dijelaskan pada **Bab 2**, **3** dan **4.** Metode identifikasi ini menerapkan amplifikasi DNA dari 6 lokus VNTR pada *S. aureus* yang dikenal sebagai "staphylococcal interspersed repeat units" atau SIRU. Keenam lokus tersebut terdiri dari SIRU01, -05, -07, -13, -15, dan -21. Jumlah unit pengulangan basa tiap lokus dihitung berdasarkan hasil amplifikasi DNA per lokusnya. Kombinasi dari jumlah unit lokus tersebut menghasilkan rangkaian angka yang kemudian dikenal sebagai profil alelik, dimana tiap profil alelik membentuk satu tipe MLVA (MT). Pada awalnya, analisa MLVA untuk *S. aureus* berupa "DNA-fingerprint", dimana hasil amplifikasi VNTR berupa pola fragmen-fragmen DNA pada gel agarosa. Pola inilah yang kemudian dipakai sebagai dasar karakterisasi apakah bakteri yang di uji berbeda satu dengan yang lainnya. Berbeda dengan yang dijelaskan diatas, metode MLVA yang kami kembangkan menggunakan pendekatan baru, yaitu skema MLVA yang berdasarkan karakter. Dimana penginterpretasian MLVA berupa profil alelik. Selanjutnya, MLVA divalidasi dan dibandingkan dengan teknik pengidentifikasian lainnya seperti, Pulsed-Field Gel Electrophoresis (PFGE), multilocus sequence typing (MLST), dan identifikasi berdasarkan protein A dari *S. aureus* yang dikenal sebagai staphylococcal protein A (*spa*)-typing.

MLVA dievaluasi berdasarkan kemampuan identifikasi (typeability), reprodusibilitas, stabilitas, kemampuan untuk membedakan atau mendiskriminasi spesies (discriminatory power), dan keselarasan secara epidemiologi (epidemiologic concordance). Sebagai kunci utama dari metode pengidentifikasian adalah kemampuan untuk membedakan spesies bakteri yang satu dengan lainnya, yang diukur berdasarkan index keragaman dari Simpson (Simpson's index of diversity). Sementara keselarasan antara dua metode identifikasi yang berbeda ditentukan berdasarkan Adjusted Rand index dan koefisien Wallace (Wallace's coefficient).

Bab 2 mendeskripsikan uji MLVA terhadap 100 *S. aureus* yang berasal dari manusia. Isolat tersebut dipilih secara random dari European Network for Antimicrobial Resistance and Epidemiology (ENARE) yang sebelumnya telah diketahui genotipenya berdasarkan MLST. Validasi MLVA ini dilakukan pada 50 isolat koleksi dari Bagian Higiene dari University Medical Centre Utrecht, yang merupakan representatif dari isolat perjangkitan dan isolat yang tidak berhubungan secara epidemiologi. Bagian Higiene melakukan pengelompokkan isolat tersebut berdasarkan phagetyping dan PFGE. Pengujian stabilitas MLVA

dilakukan pada 14 isolat yang berasal dari 4 pasien, dimana sampling dilakukan dalam kurun waktu 5 tahun.

Metode MLVA ini memperlihatkan kemampuan identifikasi yang memuaskan. Hal ini terbukti dengan teramplikasinya keenam lokus VNTR pada semua isolat yang diuji. Validasi MLVA menunjukkan keselarasan hasil dengan pengelompokkan isolat oleh Bagian Higiene, dimana MLVA dapat membedakan isolat yang berasal dari perjangkitan dan isolat yang ditemukan secara insidentil. Kami menemukan pula bahwa beberapa isolat dapat disalahartikan dan dimasukkan kedalam satu grup perjangkitan yang sama, bila pengelompokkan isolat dilakukan berdasarkan phagetyping dan PFGE. Padahal pada kenyataannya, isolat tersebut memiliki MT yang berbeda. Studi ini mendemonstrasikan pula stabilitas jangka panjang dari MLVA dan reprodusibilitasnya yang sangat baik. Hasil pengamatan ini didukung oleh lokus SIRU21 yang juga merupakan bagian dari *spa*-typing. Kami menemukan bahwa jumlah unit pengulangan basa pada lokus SIRU21 selalu selaras dengan jumlah unit pengulangan basa yang ditemukan pada *spa*-typing. Perlu diingat bahwa penentuan *spa*-typing adalah berdasarkan hasil sekuensing DNA dari protein A *S. aureus*, dengan jumlah unit pengulangan basa sebesar 24 pasang basa.

Kemampuan MLVA untuk membedakan spesies satu dengan lainnya sangat baik dibandingkan dengan MLST dan *spa*-typing, dan sebanding dengan PFGE. Adjusted Rand index dan koefisien Wallace mengindikasikan bahwa MLVA dapat memprediksi MT dan *spa* dengan baik, tapi tidak sebaliknya. Sedangkan kemampuan memprediksi dari MLVA terhadap PFGE dan sebaliknya sangatlah kurang. Hal ini menunjukkan bahwa keduanya tidak didasarkan pada sinyal genetika yang sama. Lebih jauh lagi, kami mengobservasi variasi pada satu atau beberapa lokus pada isolat dengan profil PFGE yang identik. Dalam mengatasi hal ini, kami menggunakan pembagian kriteria yang sama dengan MLVA untuk enterococcus: Tidak dapat dibedakan: bila isolate mempunyai MT yang identik; Berhubungan dekat: bila ditemukan variasi pada satu lokus (single locus variant atau SLV); Kemungkinan berhubungan: ditunjukkan dengan variasi pada dua lokus (double locus variants atau DLV); Tidak berhubungan: apabila ditemukan perbedaan lebih dari dua lokus. Perlu digarisbawahi bahwa, untuk menentukan satu isolat termasuk grup perjangkitan atau tidak, seharusnya selalu disertai dengan data epidemiologi isolat tersebut.

Oleh karena MLVA ini mampu menunjukkan potensi yang sangat besar sebagai teknik identifikasi yang cepat dan terpercaya, kami memperluas penggunaan metode ini pada MRSA dan *S. aureus* yang rentan terhadap antibiotik methicillin (methicillin-susceptible *S. aureus* atau MSSA) yang berasal dari hewan ternak (**Bab 3** dan **Bab 4**).

Bab 3 menjelaskan identifikasi genotipe pada 85 *S. aureus* yang berasal dari sapi yang menderita mastitis. Teknik identifikasi yang digunakan adalah MLVA, PFGE, MLST dan *spa*-typing. Isolat yang digunakan tersebut diatas adalah koleksi dari Fakultas Kedokteran Hewan Universitas Utrecht (UU), yang terdiri dari 30 isolat berasal dari provinsi Utrecht mewakili regional dan 50 isolat berasal dari beberapa provinsi di Belanda yang merupakan representatif nasional. 90% dari isolat berasal dari peternakan yang berbeda. Bila 2 isolat berasal dari peternakan yang sama, sampling dilakukan pada waktu yang berbeda, dengan jarak minimal satu tahun. Dengan demikian, representasi yang berlebihan dari peternakan tertentu dapat dihindari.

MLVA menunjukkan kemampuan identifikasi yang memuaskan, walaupun lokus SIRU05 sama sekali tidak terdeteksi, ditambah dengan SIRU07 dan 13 yang tidak selalu dapat diamplifikasi. Hal yang berbeda diperlihatkan oleh SIRU21 yang menunjukkan variasi cukup besar dari jumlah unit pengulangan basa. Dengan demikian, SIRU21 merupakan lokus yang menentukan pada MLVA untuk kelompok isolat ini. Pada studi ini tidak ditemukan adanya perbedaan yang antara isolat yang mewakili regional dan nasional. Sehingga bias dikarenakan perbedaan secara geografi dapat ditiadakan. Derajat kemampuan untuk mendiskriminasi spesies yang tertinggi diperlihatkan oleh PFGE, diikuti kemudian dengan MLST, MLVA dan *spa*-typing. Adjusted Rand index dan koefisien Wallace menunjukkan kurangnya kemampuan prediksi MLVA terhadap PFGE dan MLST. Hal ini secara tidak langsung mendukung pernyataan sebelumnya bahwa, MLVA mempunyai kemampuan yang kurang dalam membedakan spesies dibandingkan dengan MLST dan PFGE. Tidak terdeteksinya SIRU05 pada semua isolat mungkin dapat menjelaskan rendahnya daya diskriminasi dari MLVA.

Analisa secara mendalam pada daerah disekitar lokus SIRU05 memperlihatkan perbedaan elemen genetik yang berbeda, menjelaskan mengapa SIRU05 tidak dapat diamplifikasi. Pada *S. aureus* yang berasal dari manusia, elemen genetik pembawa sifat resisten terhadap fosfomycin terletak didekat SIRU05. Sedangkan pada *S. aureus* yang berasal dari sapi, elemen tersebut tidak terdeteksi. Perlu

diingat bahwa amplifikasi SIRU05 menggunakan primer yang didesain berdasarkan *S. aureus* yang berasal dari manusia. Lebih jauh lagi, terdapat mutasi satu nukleotida pada elemen pembawa kode genetic LysR, yang letaknya di hulu dari lokus SIRU05. LysR merupakan salah satu anggota dari famili protein regulator. Mutasi tersebut diatas, menghasilkan "stop-codon" yang mengakibatkan inaktifnya proses translasi domein substrat dari LysR. Hal inilah yang mengarah pada perbedaan pola ekspresi protein antara *S. aureus* yang berasal dari manusia dan sapi.

Studi populasi yang dilakukan dengan menggunakan teknik MLST memperlihatkan bahwa, populasi *S. aureus* yang berasal dari sapi berbeda dibandingkan dengan yang berasal dari manusia, dengan pengecualian pada 4 isolat. Keempat isolat tersebut merupakan bagian dari 'clonal complex' 97 atau CC97 yang sebelumnya ditemukan pada isolat berasal dari manusia. Penemuan ini mendukung hipotesa 'host-specificity' dari klon MRSA dan dapat mengurangi kemungkinan terjadinya transmisi bakteri dari manusia ke sapi. Yang lebih menarik lagi adalah dengan ditemukannya tipe MLST (ST) yang baru, yang merujuk pada CC yang berbeda dan belum pernah dilaporkan sebelumnya. Hal ini mengindikasikan munculnya MSSA klon baru. Sebagai tambahan, studi ini memperlihatkan bahwa *spa*-typing bukan metode yang tepat untuk digunakan pada *S. aureus* yang berasal dari sapi, dikarenakan sangat rendahnya variasi tipe *spa* yang dihasilkan dan daya diskriminasi spesies yang kurang dibandingkan MLST.

Pada **Bab 4**, genotipe dari 50 MRSA ST398 koleksi dari Fakultas Kedokteran Hewan Universitas Utrecht ditentukan berdasarkan MLVA dan *spa*-typing. PFGE dengan menggunakan enzim restriksi *Sma*I tidak mungkin dilakukan pada isolate ini, dikarenakan adanya mutasi pada tempat pengenalan restriksi.

Hasil MLVA memperlihatkan bahwa semua lokus dapat diamplifikasi kecuali SIRU05, suatu fenomena serupa dengan yang ditemukan pada **Bab 3**. Kemampuan MLVA untuk mendiskriminasi spesies sangat baik dibandingkan *spa*-typing. Hasil yang menarik adalah amplifikasi dari lokus SIRU13 pada semua isolate menghasilkan dua fragmen DNA. Hal ini ditemukan sebelumnya pada dua isolate *S. aureus* yang berasal dari manusia yang mempunyai riwayat kontak dengan peternakan babi (**Bab 2**). Berdasarkan hasil analisis sekuen DNA dari fragmen ganda tersebut diatas, jumlah unit pengulangan basa SIRU13 ditentukan berdasarkan fragmen yang terkecil. Lebih jauh lagi, investigasi dari

struktur genetik disekitar SIRU13 memperlihatkan duplikasi fragmen berukuran 162 pasang basa di daerah hulu dari lokus SIRU13 sejati. Struktur genetik yang berada di hilir SIRU13 adalah gen *hmrB.* Gen ini bertanggung jawab dalam mengkonversi ekspresi resisten terhadap methicillin dari heterogen menjadi homogen. Hipotesa kami adalah SIRU13 terlibat dalam regulasi resistensi *S. aureus* terhadap antibiotika methicillin. Walaupun semua isolat yang diuji memiliki ST yang identik, MLVA membuktikan kemampuan diskriminasi yang lebih tinggi dibandingkan *spa*-typing dan mampu memprediksi tipe *spa* dengan sangat baik.

Bab 5 menjelaskan investigasi terhadap gen yang bertanggung jawab terhadap sifat virulensi dari *S. aureus*. Isolat yang digunakan pada studi di sama dengan yang digunakan sebelumnya pada **Bab 3**. Gen virulensi yang dideteksi termasuk 19 gen toksin, 12 gen adhesion, 3 gen yang terlibat dalam 'immune evasion', dan 6 gen yang lain. Cara pendeteksiannya adalah dengan menggunakan PCR. Kehadiran dan ketidakhadiran gen virulensi membentuk profil gen virulensi. Seluruh isolat yang diuji kemudian dikelompokkan berdasarkan profil tersebut, dan menghasilkan lima profil utama dari gen virulensi. Hasil pengelompokkan ini dibandingkan dengan empat metode pengidentifikasian seperti yang disebutkan di **Bab 3**. Disini kami mengobservasi persesuaian antara pengelompokkan berdasarkan profil gen virulensi dan keempat metode identifikasi PFGE, MLVA, *spa*-typing dan MLST, dengan pengecualian pada 2 grup dikarenakan adanya bovine-specific *S. aureus* pathogenicity island atau SaPIbov. Penemuan ini mengindikasikan mobilisasi SaPIbov yang independen. Dari seluruh gen virulensi yang teramplifikasi, kemudian disaring seperangkat gen yang selalu terdeteksi di semua isolate. Gen tersebut adalah β-hemolysin (*hlb*), elastin-binding protein (*ebps*), serine protease (*sspA*), cysteine protease (*sspB*), fibronectin-binding protein A (*fnbA*), dan coagulase (*coa*). Keenam gen ini nampaknya memegang peranan penting dalam pengekspresian mastitis pada sapi. Tetapi bagaimanapun juga, hal ini tidak menutup kemungkinan gen lain untuk mengkontribusi keparahan dari penyakit tersebut.

Bab 6 menjelaskan transmisi MRSA diantara peternakan babi yang dipilih secara random di Belanda, termasuk 12 'narrowing farm', 11 'finishing farm', 6 'farrow-to-finish', dan 1 'rearing farm'. Dua puluh lima peternakan berasal dari provinsi Utrecht, dan enam lainnya berasal dari beberapa provinsi di Belanda. Setiap penggunaan antibiotika pada tiap peternakan dicatat, dan sampling juga dilakukan terhadap peternak dengan dasar sukarela. Sebagai tambahan adalah 6 'supplying farm' untuk diinvestigasi apakah MRSA diperoleh dari pembelian

ternak yang sudah terkolonisasi. Sampling tidak dapat dilakukan pada satu peternakan dikarenakan peternakan tersebut adalah “close-farm”. Pada studi ini, dilakukan uji kerentanan terhadap antibiotika, identifikasi genotipe PFGE dengan menggunakan *Sma*I, MLST, *spa*-typing dan SCC*mec* typing.

Seperti yang ditelah dikemukakan pada **Bab** 4, PFGE dengan menggunakan *Sma*I tidak dapat dilakukan pada MRSA isolat yang berasal dari babi dan manusia yang mempunyai riwayat kontak dengannya. Hal ini disebabkan adanya modifikasi pada situs pengenalan *Sma*I.

Bukti bahwa MRSA dapat diperoleh dari perternakan pensuplai diperlihatkan oleh tipe *spa* yang identik antara peternakan pensuplai dan pembeli. Tetapi MRSA juga dapat ditemukan pada peternakan tertutup (close-farm), yang menunjukkan bahwa penyebaran MRSA tidak hanya disebabkan oleh masuknya ternak yang sudah terkolonisasi sebelumnya. Pekerja pada peternakan dimana ditemukannya MRSA mempunyai resiko yang tinggi untuk terkolonisasi. Sebagai tambahan, kami menemukan MRSA dengan tipe *spa* yang identik tetapi berbeda tipe SCC*mec* di dua peternakan. Keadaan ini mengindikasikan insersi elemen genetik SCC*mec* yang berbeda pada MSSA dengan genotipe yang sama.

Pola resisten dari MRSA yang kami deteksi adalah T, TE, TEL, TKG dan TELKG (T: tetracycline; E: erythromycin; L: lincomycin; K: kanamycin; G: gentamicin). Tiga peternakan menunjukkan dua pola resistensi yang berbeda (T dan TGK; T dan TEL). Penemuan ini sesuai dengan penggunaan oxytetracycline pada peternakan tersebut. Dengan demikian, penggunaan antibiotik oxytetracycline nampaknya merupakan faktor resiko terhadap ditemukannya ternak yang positif membawa MRSA. Lebih jauh lagi, studi ini tidak mendeteksi adanya gen toksin. Kami berasumsi bahwa akuisisi dari satu atau lebih gen toksin dapat meningkatkan patogenisitas. Sebagai tambahan, penyebaran klonal dipertegas dengan kenyataan bahwa semua isolat yang digunakan adalah MRSA ST398 yang memiliki lima tipe *spa* yang berhubungan dekat. Sayangnya pada saat studi ini dilakukan, metode MLVA yang kami jelaskan pada Bab 2 belum tersedia.

Bab 7 menjelaskan tentang penggunaan teknologi microarray dengan jumlah target (probe) yang terbatas untuk mengenali perjangkitan dan studi populasi dari *S. aureus* yang berasal dari manusia. Isolat yang digunakan pada Bab 2 digunakan pada studi ini, ditambah dengan 157 isolat yang berasal dari 6 ICU di

Eropa. Identifikasi genotipe dari semua isolat pun dilakukan sebagai perbandingan.

Studi memperlihatkan bahwa semua isolat dapat dihibridisasi. Walaupun beberapa isolat menghasilkan sinyal yang rendah, tetapi isolat tersebut tetap diperhitungkan dalam analisa. Profil microarray (AP) dihasilkan oleh kombinasi kehadiran dan divergensi dari probe. Pengelompokkan AP ditentukan oleh titik pintas 'genetic distance' atau GD dari replikat isolat yang diuji. Dua macam titik pintas GD digunakan untuk tujuan yang berbeda. Pertama, untuk mengetahui adanya perjangkitan. Kedua, untuk menganalisa struktur populasi.

Evaluasi dari microarray memperlihatkan bahwa metode ini mempunyai daya identifikasi dan reprodusibiliti yang baik, dan stabil. Kemampuan microarray untuk mendiskriminasi spesies sama dengan MLVA. Analisa keselarasan secara epidemiologi menunjukkan bahwa microarray dapat memprediksi MLST dan tipe *spa* dengan logis. Dan yang paling penting adalah microarray mampu mengklasifikasi isolat dari perjangkitan dengan benar, walaupun terdapat beberapa pengecualian. Hal ini kemungkinan disebabkan oleh variasi minor pada gen asesoris yang tidak stabil. Dimana gen tersebut mudah diperoleh dan juga mudah hilang, walaupun pada bakteri yang secara genetik berhubungan dekat. Lebih jauh lagi, tidak dapat dipungkiri adanya kemungkinan salah penafsiran pada saat pengelompokkan isolat oleh Bagian Higiene sehubungan dengan isolat perjangkitan atau isolat yang secara genetik tidak berhubungan (Bab 2). Sebagai tambahan, kami mengobservasi bahwa microarray mampu memprediksi hasil dari MLST dengan logis, sehingga dapat digunakan secara luas untuk studi populasi.

Penyaringan jumlah probe yang signifikan ditentukan dengan bantuan perhitungan statistik Significance Analysis for Microarray (SAM). Hasil analisa memperlihatkan bahwa pada kedua kelompok isolate, penggunaan 205 probes memberikan hasil yang sama dengan microarray yang menggunakan 400 probes. Adapun hal hal yang perlu ditingkatkan pada metode ini adalah penentuan titik pintas GD dan eliminasi probes yang tidak dapat direproduksi hasilnya. Penambahan probe yang spesifik misalnya, faktor-faktor virulensi dan genetik elemen yang mengkode kebal antibiotik, dapat meningkatkan kemampuan identifikasi dari microarray.

Sebagai kesimpulan, metode MLVA yang kami kembangkan ini sangat baik untuk mempelajari epidemiologi dari MRSA dan MSSA yang berasal dari manusia dan hewan. Metode ini cepat, murah, mempunyai daya diskriminasi spesies yang tinggi, dapat direproduksi, stabil dan portable. Sehingga dapat dipergunakan secara luas pada institusi dengan keterbatasan akses untuk fasilitas sekuensing DNA. Penggunaan metode ini akan lebih meluas lagi dengan tersedianya web-based database dari MLVA, yang memungkinkan perbandingan dan pertukaran informasi secara internasional.

Microarray dengan menggunakan probes yang terbatas dapat digunakan untuk mengidenifikasi perjangkitan dan studi populasi dari *S. aureus*. Kualitas dan penggunaan metode ini dapat ditingkatkan dengan penentuan titik pintas GD yang optimum, eliminasi probe yang tidak stabil, dan penambahan probe yang lebih spesifik.

Gen tertentu tampaknya bertanggung jawab dalam patogenesis mastitis pada sapi. Penjelasan alternative adalah, bahwa perbedaan profil gen dapat menghasilkan penampakan mastitis yang berbeda. Selanjutnya, studi pada MRSA ST398 memperlihatkan bahwa transmisi MRSA antar peternakan dapat terjadi.

Acknowledgment

It is a pleasure to thank many people who made this thesis possible.

Foremost, I would like to express my sincere gratitude to my promotor, Prof. Dr. Jan Verhoef, for the wonderful opportunity to pursue my career in research at the Department of Medical Microbiology University Medical Centre Utrecht. Your guidance, encouragement and untiring support were invaluable.

My sincere gratitude to Dr. A.C. Fluit. Dear Ad, I am so grateful to have you as my co-promotor.Your enthousiasm, inspiration, and great efforts to explain things clearly and simply, have taught me the incredible art of bacteria. The work was though, but the impact and influence have given me is one of the best ever. Throughout my thesis-writing period, you provided encouragement, sound advice, lots of good ideas, and thank you for your patience. I never thought that I could be where I am today. Because of you, I survived.

I am indebted to Prof. Dr. Sangkot Marzuki who gave me a chance that might not come twice. The Eijkman Institute in Jakarta, the place where I began my research career, has provided me an invaluable knowledge and experience.

Special gratitude goes to Prof. Dr. Irawan Yusuf at the Hasanuddin University School of Medicine in Makassar. I have been fortunate to have you as my teacher and my mentor. Your insight and knowledge have attracted me to the world of research.

Moreover, I wish to express my cordial appreciation to Prof. Dr. Dik Mevius, Dr. Engeline van Duijkeren, Dr. Frank H. Schuren, and Dr. Rob J.L. Willems for your fruitful scientific collaborations. Your comment, suggestion and burning question were always stimulating and inspiring.

To the members of the reading committee, Prof. Dr. I.M. Hoepelman, Prof. Dr. D.J. Mevius, Prof. Dr. J.P.M. Tommassen, Prof. Dr. A. van Belkum, and Prof. Dr. J. Wagenaar, I would like to thank you very much for your time, expertise and support. I look forward to discuss my thesis with you during my defense.

During this work, I have collaborated with many colleagues for whom I have great regard. The *S. aureus* group: Bob Bloemendaal, Adrienne Box, Ellen Brouwer and Marc Jansen, thank you for all supports and contributions for our manuscripts. Beste Ellen en Marc, mijn paranimfen, enorm bedankt voor de geweldige ondersteuning van de promotie. My thanks also go to Edwin Boel, Nicole Smits, and Maarten. Dear Neeltje, I always enjoy our chitchat ☺ Thank you for the wonderful friendship and I wish you all the best for your promotion.

My deepest appreciation goes to Martien Caspers (TNO Zeist) for the time and efforts you have given. Through you, I learnt what and how the microarray from scratch. Your expertise has rescued me from drowning in the ocean of microarray data. Martien, you're really the excel GURU! ☺ To Marco Jaspers, bedankt voor

de gezelligheid! By the way, I still believe that it's you on that big poster ☺. To all my labmates at TNO Zeist, thank you for the hospitality and assistance. It was short, but I had a great time and enjoyed it a lot!

Mijn oude kamergenoten, Carla, Tjomme (een bakje troost? ☺), Pauline, David (the football-guy and the historian), John (the man in black), bedankt voor de gezellige tijd! John, thanks for the story of a French lady ☺. Janetta, Martiene (Ola makaronie!), Marijke, Aletta, and Inge, jullie zijn echt gewelldig! No words can express my thanks to you all, for what we have shared in a good time and a bad time. I hope our friendship will last, no matter what. Ladies..I think it's time for another reunion again ☺. Inge, actually there was no traveling competition, but completing the "have been there" list ☺

Piet –the father of Lab G04.614- (Hoest?), Stephanie, Jelle (apakabar? Aju paraplu?), Mignon, Miranda, Willemine, Pauline, Dorien, Wat een gezellig! ☺ Bertie (thanks for helping me with the first project), Machiel, Louis en Martin, Jos en Gerrit (sorry for sometimes breaking the "openingstijd" rule), Aafke and Judith (for PFGE), Henk en Loek (the computer guys), bedankt voor de fantastische samenwerking.

Helen, I wish you and Marc will have a great time in Indonesia. Jovi, thanks for all the info and good luck with your new job. We'll keep in touch! ☺ Miquel, thanks for the friendship, your guidance on my first year, and for the book of course! I still have to finish it though ☺ Harm and Yfke, thank you for your assistance and support. The AIO room crews: András, Bas, Antoni, Dodi, Xinglin, Marieke, Armand, Ilse, thanks for welcoming me during my "nomad" period.

I wish to extend my warmest thanks to my other friends. Alexey Rodriguez, thanks for sharing the successes and frustrations of the PhD life ☺. Iwan Takwin, thanks for helping me prepared the cover. The "U30" friends who almost always brightened-up my day, Bernadetta 'Detty' Budhawara (thanks for providing me a shelter, I know I can count on you), Irene 'Ririn' Hadiprayitno (finally, the status affirmed! ☺), Rizki 'aa QQ' Permana (you're time is coming soon..don't worry, be happy ☺), and Raden 'Wahyu' Wahyuningrat-the journalist (koffie verkeerd or coffee for kids? ☺).

Prof. Jo Marx and Zwaniek, thank you for your support, hospitality, and all the cultural activities. I really had a great time!

My heartiest thanks to my Dutch parents, Henny and Jan van Kats, for your kindness and affection, and for never letting me feel that I am away from my family. You gave me support and untiring help during my difficult moments. Harstike bedankt voor alles. Jullie zijn echt super geweldig!

Finally, I would like to thank everybody who was important to the realization of this thesis, as well as expressing my apology that I could not mention personally one by one.

• • •

I am nothing without my family…
to them I dedicated this thesis.

My foremost love and thankfulness to my parents. Your endless unconditional love and prayers are my strength. I can't repay the lessons you tought, I can't return the encouragement and loving words of praise. Only love that I can give, it's all the love you've earned. Mom dan Dad, my apologize for always being away from home. Papa, mama, terima kasih untuk cinta sepanjang hayat, doa tulus yang selalu dipanjatkan, dan semua pengorbanan yang tak ternilai. Dukungan papa dan mama adalah kekuatan Ika. Maafkan Ika yang selama ini selalu jauh dari papa dan mama.

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I owe my loving thanks to my husband, Buyung Afrianto. Without your love, understanding and persistent confidence in me, this work would not have been completed. Four years living in two continents apart was not easy (so, for those who read this..Don't try this at home! ☺), Thanks God we survived! Liefje, we will catch-up those missing years!

My sincere gratitude goes to my parents, brother and sister-in-law, thank you for your loving and unfailing supports.

List of publications

1. **Ikawaty, R**., R. J.L.Willems, A.T.A. Box, J. Verhoef and A.C. Fluit. A novel multiple multiple locus variable number tandem repeat (VNTR) analysis for rapid molecular typing of human *Staphylococcus aureus*. Journal of Clinical Microbiology. 2008. 46: 3147-3151.

2. **Ikawaty, R**., E.C. Brouwer, M.D. Jansen, E. van Duijkeren, D. Mevius, J. Verhoef, A.C. Fluit. Characterization of Dutch *Staphylococcus aureus* from bovine mastitis using a multiple locus variable number tandem repeat analysis. Veterinary Microbiology. 2008. doi.1.1016/j.vetmic.2008.10.034.

3. E. van Duijkeren, **R. Ikawaty**, M.J. Broekhuizen-Stins, M.D. Jansen, E.C. Spalburg, A.J. de Neeling, J.G. Allaart, A. van Nes, J.A. Wagenaar, A.C. Fluit. Transmission of methicillin-resistant Staphylococcus aureus strains between kinds of pig farms. Veterinary Microbiology. 2008. 126: 383-389.

4. E. van Duijkeren, D.J. Houwers, A. Schoormans, M.J. Broekhuizen-Stins, **R. Ikawaty**, A.C. Fluit, J.A. Wagenaar. Transmission of methicillin-resistant *Staphylococcus intermedius* between humans and animals. Veterinary Microbiology. 2008. 128: 213-215.

5. Yusuf, I., Djojosubroto, M.W., **Ikawaty, R**., Lum, K., Kaneko, A., Marzuki, S. Ethnic and geographical distributions of CYP2C19 alleles in the populations of Southeast Asia. Advances in Experimental Medicine and Biology. 2003. 531: 37-46.

Author Bio

Risma Ikawaty was born on February 23rd, 1975 in Kediri, Indonesia. She graduated high school (SMA Negeri 2 Makassar) in 1993. In the same year, she attended the Hasanuddin University School of Medicine in Makassar, after which she completed her medical doctor degree with cum laude in 2000. Soon after, Risma joined the Eijkman Institute for Molecular Biology in Jakarta where she evolved her research career. At the end of 2002, she was invited by Prof. Dr. Jan Verhoef and started her doctoral program at the Department of Medical Microbiology University Medical Center Utrecht in The Netherlands. She commenced work in the group of *Staphylococcus aureus* under supervision of Prof. Dr. Jan Verhoef and Dr. A. C. Fluit at the end of 2004. Upon finishing her PhD program, Risma plans to continue her career in the field of infectious diseases and molecular biology.

Appendix

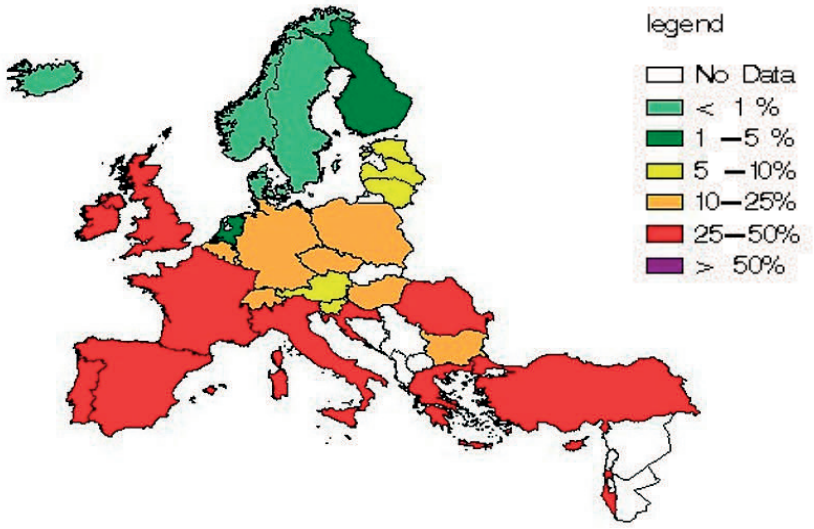

Figure 1. Proportion of MRSA isolates in 33 participating countries in Europe in 2007 reported by the European Antimicrobial Resistance Surveillance System (EARSS). (http://www.rivm.nl/earss/).

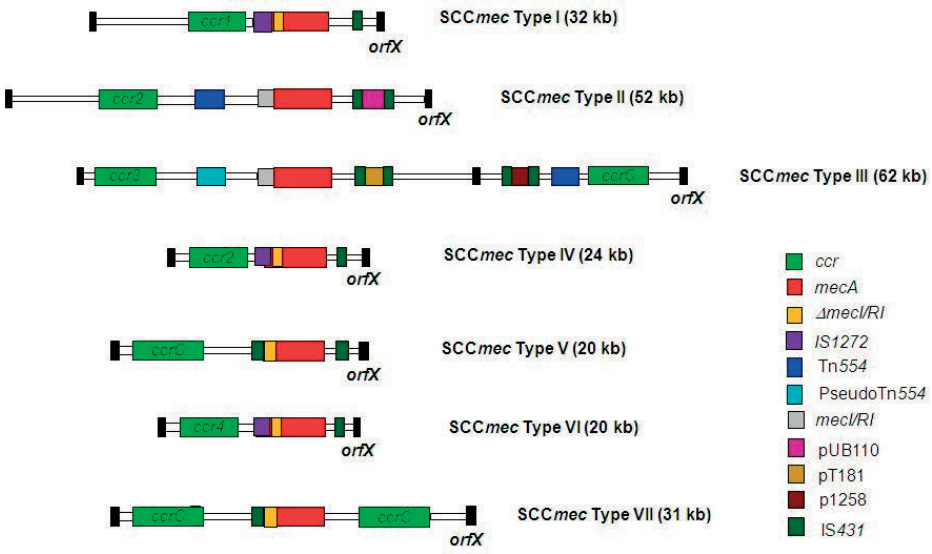

Figure 2. A schematic drawing of the seven SCC*mec* types (I-VII). All the SCC*mec* cassettes are integrated in a conserved open reading frame (*orfX*). Colored boxes represent the major elements of each SCC*mec* cassette.

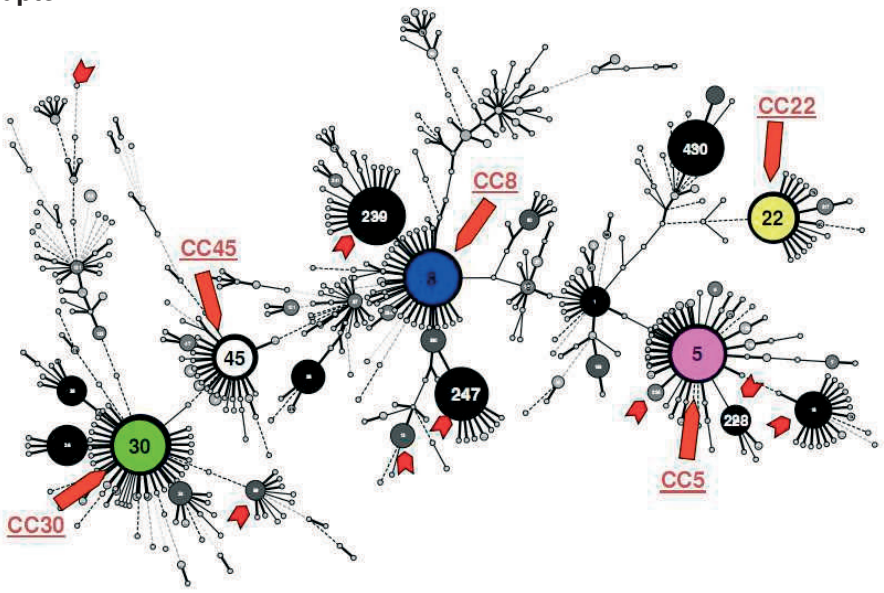

Figure 1. Minimum spanning tree of the *S. aureus* population structure based on MLST. Each circle represents a different ST. The number in the circle indicates the ST. Orange arrow indicates the distribution of samples used in this study. Thus it is obvious that they were well distributed, represented the five major clonal complexes (CC5, CC8, CC22, CC30 and CC45) and singletons.

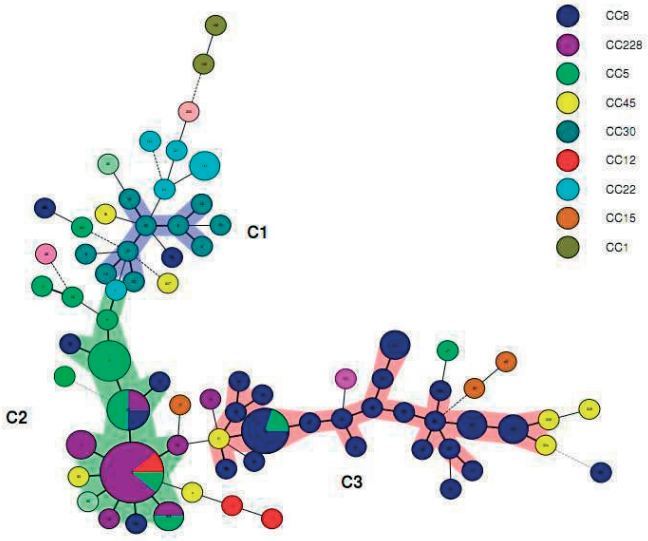

Figure 2. Population structure of *S. aureus* isolates based on MLVA. Each circle represents a different MLVA profile. Three clusters (C1, C2, and C3) were identified as shown by the three colors (blue, green, and pink). Heavy lines connecting two MTs denote a single-locus variant, thin lines denote MTs with a double-locus variant, and dotted-lines connect MTs that differ by more than two loci.

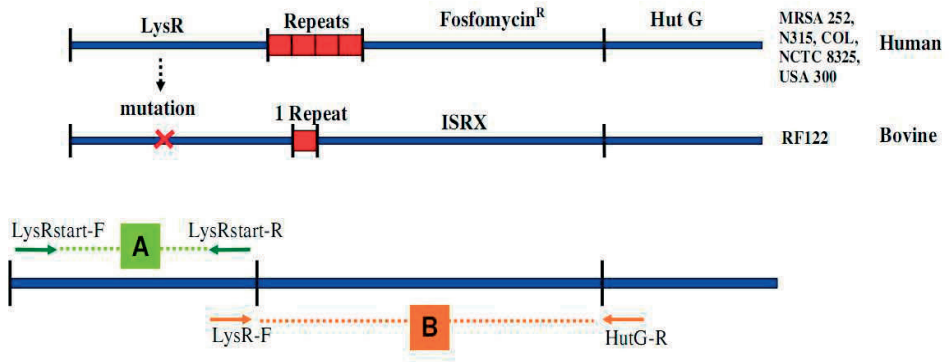

Figure 1. Strategy for analyzing the region bordering the SIRU5 locus. The bovine *S. aureus* (BSA) RF122 reference strain showed a different genetic structure compared to human *S. aureus* reference strains. The genetic element at the right of the repeat region consists of a fosfomycin resistance gene in human *S. aureus* and was replaced by insertion site region X (IS*RX*) in RF122. A single nucleotide mutation in *lysR* gene region was present in RF122. The primers were designed to detect the presence/absence of a single nucleotide mutation (fragment A) by DNA sequencing and the presence of fosfomycin resistance gene or IS*RX* structure (fragment B).

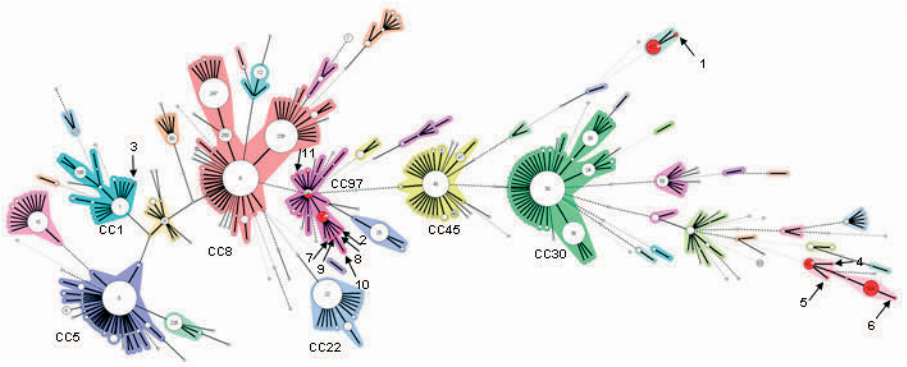

Figure 3. Minimum spanning tree of *S. aureus* based on MLST. Five major clonal complexes are present within the *S. aureus* population: CC5, CC8, CC22, CC30 and CC45. Each circle represents a different MLST type. Single locus variant STs are connected by a thick line, double locus variant STs by a thin line, triple locus variant STs by a dark grey dashed line, and STs with more than 3 loci variant are connected by a light grey dashed line. Red circles indicate *S. aureus* isolated from bovine mastitis. Arrows indicated new MLST types observed in this study: 1: ST 1118; 2: ST 1119; 3: ST 1121; 4: ST 1122; 5: ST 1123; 6: ST 1124; 7: ST 1125; 8: ST 1126; 9: ST 1127; 10: ST 1128; 11: ST 1129.

Chapter 4

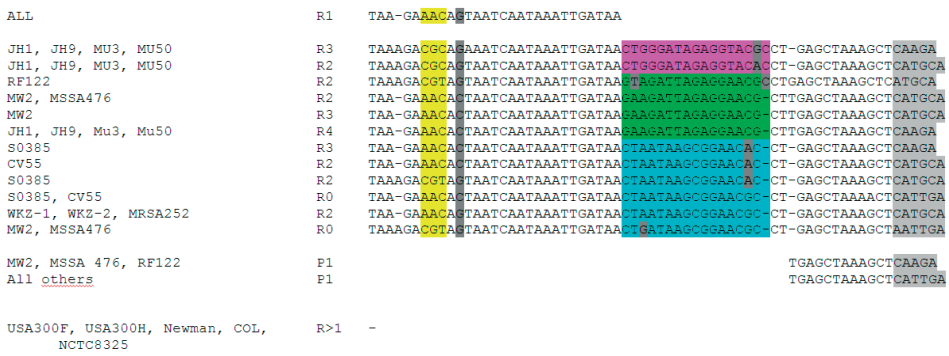

Figure 3. Comparison of the repeat sequences of SIRU13. Also included the sequences of P1. Colors and gray indicate variable sequences. A hyphen indicates a lacking nucleotide.

Chapter 5

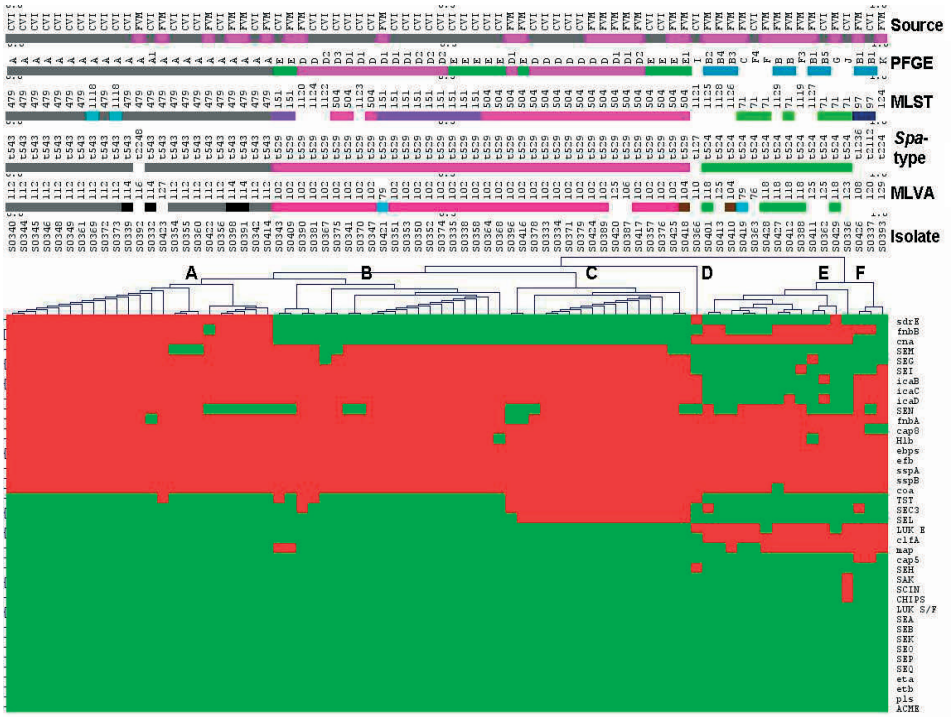

Figure.Virulence factor profiles observed within various typing methods. Source: Place where the isolates were taken. FVM: Veterinary Faculty Utrecht University; CVI: Central Veterinary Institute, Lelystad, The Netherlands. The presence and absence of genes indicated by red and green spots.Virulence factor profiles were defined based o the presence and absence of the genes, and resulted 5 main profiles, A, B, C, D, and E.

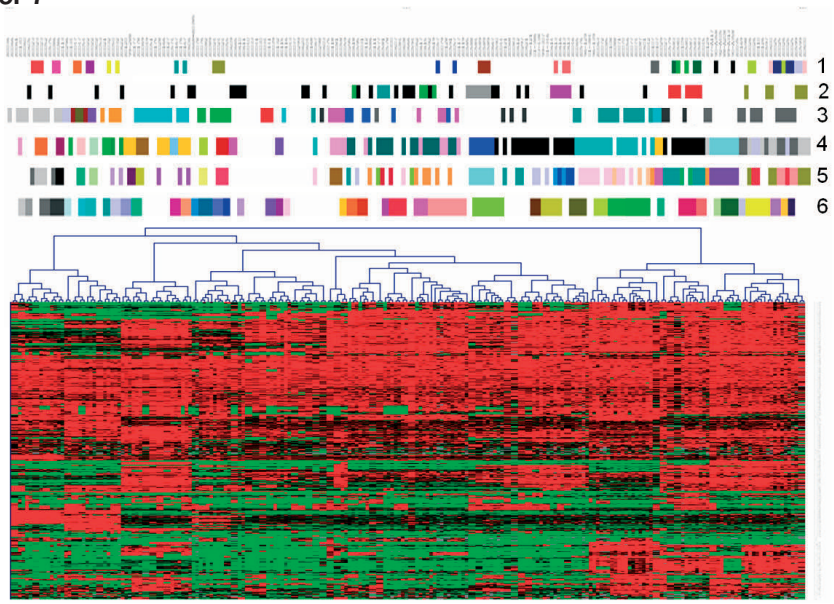

Figure 1. Two dimensional hierarchical clustering of the 164 *S. aureus* strains obtained from hybridization with 400 probes. Red and green spots represented the presence and divergence of genes (probes).The y-axis shows the 400 probes, while the isolates are on the x-axis. Clustering of the isolates 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 (see also the text for examples).

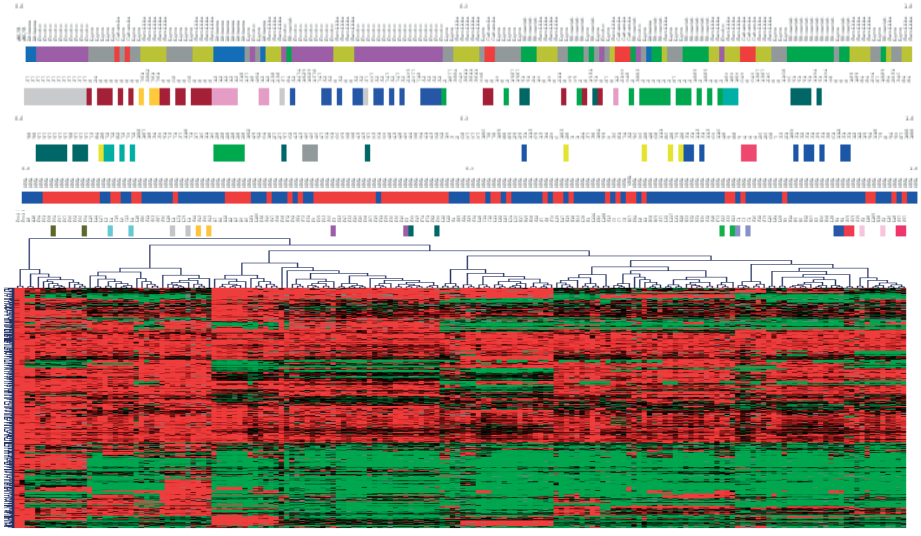

Figure 2.Two dimensional hierarchical clustering of 157 ICUs isolates using 400 probes. Shown from top to bottom clustering based on city of origin, *spa*-type, MLVA-type, MRSA/MSSA and duplicates.

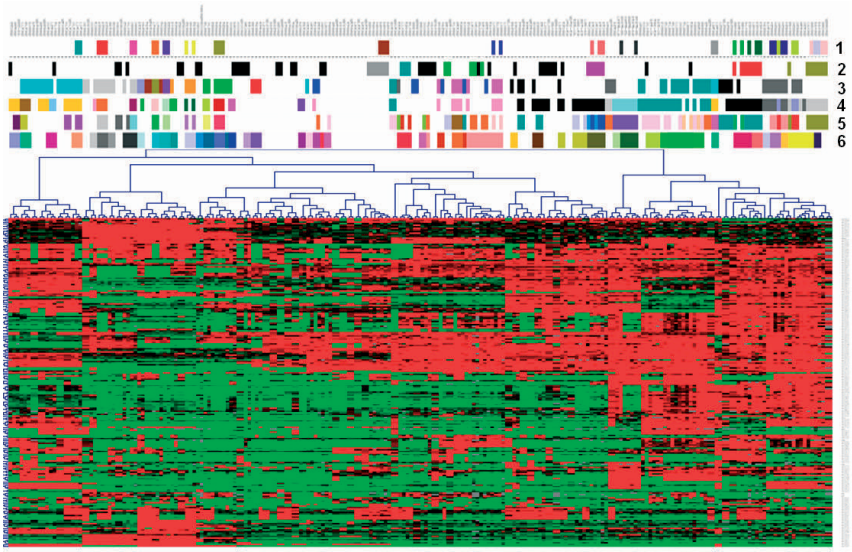

Figure 3. Two dimensional hierarchical clustering of 164 isolates with 205 probes after 45% of probes reduction (cut-off GD 6.6). Clustering of the isolates was determined based on (1) replicates; (2) epidemiological relatedness; (3) MLST types; (4) *spa*-types; (5) MLVA types; and microarray profile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

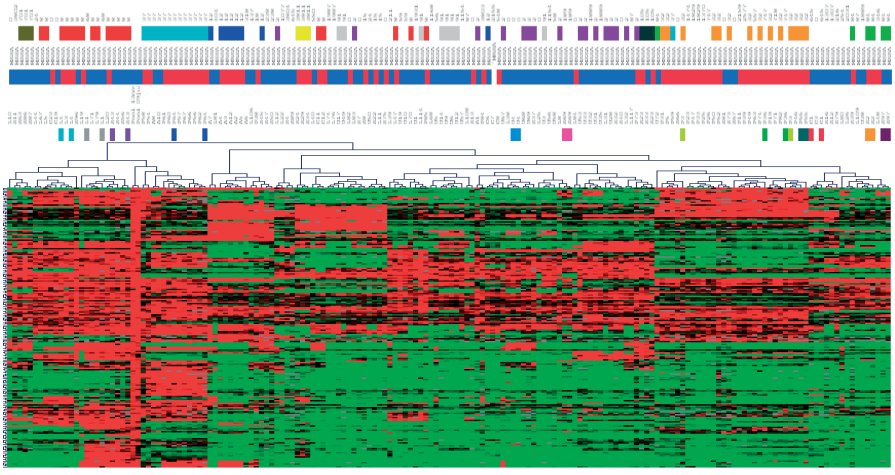

Figure 4. Two dimensional hierarchical clustering of 157 ICUs isolates using 205 probes. Shown from top to bottom clustering based on *spa*-type, MRSA/MSSA and duplicates.

Errata

1. Page 14. Table 1. Tn*554* encodes erythromycin and spectinomycin resistance.
2. Page 77. On Figure 2, dendrogam was followed by ID of isolate, MLST type, *spa*-type, and MLVA profile.
3. Page 91. MLVA typing of 50 isolates resulted in 6 dominant MTs.
4. Page 92.
   Table 1.

No.	Sample	Source	Prov.[a]	*Spa*-type	MLVA type	SIRU[b]					
						01	05	07	13[c]	15	21
1	S0623	Pig	NB	t567	413	3	999	3	3	2	5
2	S0624	Pig	NB	t567	413	3	999	3	3	2	5
3	S0625	Pig	NB	t567	413	3	999	3	3	2	5
4	S0626	Pig	NB	t567	413	3	999	3	3	2	5
5	S0627	Pig	NB	t567	413	3	999	3	3	2	5
6	S0628	Pig	NB	t567	413	3	999	3	3	2	5
7	S0629	Pig	NB	t567	413	3	999	3	3	2	5
8	S0696	Pig	NB	t567	413	3	999	3	3	2	5
9	S0697	Pig	NB	t567	413	3	999	3	3	2	5
10	S0698	Pig	NB	t567	413	3	999	3	3	2	5
11	S0699	Pig	NB	t567	413	3	999	3	3	2	5
12	S0700	Farmer	NB	t567	413	3	999	3	3	2	5
13	S0701	Farmer	NB	t567	413	3	999	3	3	2	5
14	S0630	Pig	NB	t567	428	3	999	3	2	2	5
15	S0631	Pig	NB	t567	428	3	999	3	2	2	5
16	S0632	Farmer	NB	t567	428	3	999	3	2	2	5
17	S0633	Farmer	NB	t567	428	3	999	3	2	2	5
18	S0702	Farmer	NB	t567	428	3	999	3	2	2	5
19	S0703	Farmer	NB	t567	428	3	999	3	2	2	5
20	S0635	Pig	NB	t108	429	3	999	3	2	2	6
21	S0636	Pig	NB	t108	429	3	999	3	2	2	6
22	S0637	Pig	NB	t108	429	3	999	3	2	2	6
23	S0638	Pig	NB	t108	414	3	999	3	3	2	6
24	S0639	Pig	NB	t108	414	3	999	3	3	2	6
25	S0640	Pig	NB	t108	414	3	999	3	3	2	6
26	S0641	Pig	NB	t108	414	3	999	3	3	2	6
27	S0642	Pig	NB	t108	414	3	999	3	3	2	6
28	S0643	Farmer	NB	t108	414	3	999	3	3	2	6
29	S0652	Pig	NB	t108	414	3	999	3	3	2	6
30	S0653	Farmer	NB	t108	414	3	999	3	3	2	6
31	S0662	Pig	Gelder	t108	414	3	999	3	3	2	6
32	S0663	Pig	Gelder	t108	414	3	999	3	3	2	6
33	S0664	Farmer	Gelder	t108	414	3	999	3	3	2	6
34	S0644	Pig	Utr	t011	426	3	999	3	3	2	7
35	S0645	Pig	Utr	t011	426	3	999	3	3	2	7
36	S0646	Pig	Utr	t011	426	3	999	3	3	2	7
37	S0647	Pig	Utr	t011	426	3	999	3	3	2	7
38	S0648	Pig	Utr	t011	426	3	999	3	3	2	7
39	S0649	Pig	Utr	t011	426	3	999	3	3	2	7
40	S0650	Pig	Utr	t011	426	3	999	3	3	2	7
41	S0651	Pig	Utr	t011	426	3	999	3	3	2	7
42	S0655	Pig	Gelder	t899	416	3	999	5	3	2	5
43	S0656	Pig	Gelder	t899	416	3	999	5	3	2	5
44	S0657	Pig	Gelder	t899	416	3	999	5	3	2	5
45	S0659	Pig	Gelder	t899	416	3	999	5	3	2	5
46	S0660	Pig	Gelder	t899	416	3	999	5	3	2	5
47	S0661	Pig	Gelder	t899	416	3	999	5	3	2	5
48	S0634	Farmer	Utr	t011	430	3	999	3	2	2	7
49	S0654	Pig	Gelder	t1939[d]	415	3	999	5	3	2	4
50	S0658	Pig	Gelder	t899	427	3	999	4	3	2	5

5. Page 94. Two sequence motifs are found: CAAGA and CATGCA.

6. Page 97. Also the sequences of the D1 sequences are strongly conserved allowing only a few mutations (Fig. 5).

WKZ-1&2,MRSA252,CV55 (D1)
CGCAGTGGTTGACTGGTCATCCAATGGAGAAATGCCTGACCTAGTCAACTTTGCGGGGGAAATTCTAAGCAACCAAGATATGGTTCCAGAATTTCTCCC

S0385 (D2,D1)
CGCAGTGGTTGACTGGTCATCCAATGGAGAAATGCCTGACCTAGTCAACTTTGCGGGGGAAATTCTAAGCAACCAAGATAAGGTTCCAGAATTTCTCCC

CV55 (D2)
CGCAGTGGTTGACTGGTCATCCAATGGAGAAATGCCTGACCTAGTCAACTTTGCGGGGGAAATTCTAAGCAACCTAGATAAGGTTCCAGAATTTCTCCC

All others (D1)
CGCAGTGGTTGACTGGTCATCCAATGGAGAATTGCCTGACCTAGTCAACTTTGCGGGGGAAATTCTAAGCAACCTAGATAAGGTTCGAGAATTTCTCCC

Figure 5.

7. Page 146. Microarray construction. A mixed genome microarray was constructed using genomic DNA of 7 *S. aureus* isolates (Table 1)..
8. Page 149. Probes Reduction. SAM (Significance Analysis of Microarray)
9. Page 156. Reduction of probes. (Fig.3) and (Fig. 4) are moved to the last sentence.
10. Page 156. Figure 3. 49% of probes reduction instead of 45%.