Binary typing of *Staphylococcus aureus*

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Aan mijn ouders
Aan Mascha en José

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General introduction: Molecular approaches to the epidemiological characterization of *Staphylococcus aureus*

Chapter

1

W. B. van Leeuwen

Submitted for publication

1. BACTERIAL TYPING

Over the past century microbiologists have searched for more rapid and efficient means for the differentiation of *Staphylococcus aureus* strains. The discrimination between strains of *S. aureus* has principally relied on phenotypic characteristics including phage typing (5, 68). Consecutive phases (generations) in the development of molecular biology over the past decades have opened new ways for the exploitation of the genetic diversity within the *S. aureus* species (19). Molecular techniques were applied to elucidate the basic mechanisms of staphylococcal pathogenicity and to track the spread of *S. aureus* clones, especially the methicillin-resistant clones. The discrimination between isolates of a bacterial species is central to many aspects of clinical microbiology (70). Various forms of intraspecies variation are used for molecular typing and the appropriateness of a given typing method depends on the epidemiological questions that are posed and the population structure of the species under study.

1.1 Purpose of epidemiological typing.

Many epidemiological studies are concerned with relationships between strains which are isolated within a short period of time, such as for instance infectious disease outbreak investigations. Other studies, e.g. epidemiological surveillance of infectious diseases or the analysis of the population genetics, address the relationship between strains recovered over an extended period of time and over a broader geographical range.

Outbreak investigation. An outbreak is defined as a temporal increase in the frequency of colonization and/or infection by a given microorganism. Comparative typing is applied to the development of outbreak control strategies and address questions regarding the extent of epidemic spread of microbial clones, the number of clones involved in transmission and infection, the monitoring of reservoirs of epidemic clones or for the evaluation of the efficacy of control measures.

Pathogenesis. Genetic variation in *S. aureus* is very extensive. More than 20% of the genome comprises of dispensable genetic material. Large regions of difference were identified, and many of these regions harbor genes that encode putative virulence factors (16). Typing techniques that are able to detect distinct epidemiological markers within a species, in order to identify pathogenic strains within this species, provide insight into pathogenesis.

Surveillance of infectious diseases. Surveillance is defined as the ongoing systematic collection analysis, and interpretation of health data, regarding the occurrence of infectious diseases or agents. The reporting of the results of such analysis to those individuals and authorities who need to know is the ultimate goal, which in turn can be translated into preventive or therapeutic measures. Typing methods provide essential information in the epidemiological surveillance of infectious diseases. However, the selection of stable, discriminative definitive epidemiological markers for such a typing system is essential. These so-called library typing systems produce high throughput results and have a standardized scoring method and a uniform nomenclature of types. Data can be compared over time and place for diverse bacterial species and are, for that reason, essential components of surveillance studies.

Population structure. Genetic diversity within a population is generated by the accumulation of mutations. The significance of these mutations for the behavior of certain genotypes in a bacterial population (epidemicity, virulence, antibiotic resistance, etc.) can be elucidated by typing systems. In order to determine the population structure within a species, specific high-throughput typing systems are needed. Such typing data are crucial in defining the level of similarity within a given species. Data from multilocus enzyme electrophoresis (32, 56, 57) and recently from multilocus sequence typing (12, 14, 15, 40, 63), designed to detect associations between several conserved genes at different sites (loci) on the chromosome, have revealed that among bacteria different types of population structures exist. Some species were found to have a clonal structure (*Escherichia coli*, *Salmonella* sp.) because of a low rate of recombination of large chromosomal segments. The clonality of a population can be deduced by the global distribution of a single genotype. At the other extreme some species exhibit a panmictic population structure (*Neisseria gonorrhoeae*), and excessive recombination may occur between isolates. An intermediate type of population structure, the so-called epidemic structure was also found (*N. meningitidis*) (42, 43). This structure is essentially panmitic. However, occasionally a highly successful clone arises and increases rapidly in frequency to produce an outbreak.

1.2 Fundamentals of bacterial genomic variability.

The basic assumption in any typing system is that genetically related strains are descendants of a single precursor. Consequently, these descendants share characteristics that differ from those of genetically unrelated strains. The utility of such characteristics for epidemiological purposes is related to their stability within a strain and general diversity at the species level. Genetic diversity is increased by various mutational processes. These processes includes the production of spontaneous point mutations, several types of genetic rearrangements, and DNA acquisition. Information on factors that are involved in the generation of genetic variation and in the modulation of the frequency of genetic variation is scarce (4). It is a challenge for future studies to investigate the more general nature of unexpected gene activities with respect to biological evolution, which ensures biodiversity and represents a guarantee for maintenance and development of certain microbial species over a prolonged period of time. In contrast to mutation, selection factors acting on bacterial populations limit the level of diversity. The factors that enable bacteria to cause infection, generally are not uniformly distributed within a species. The organisms associated with infections often represent a smaller subset of the many strains or genotypes that constitute a species. Consequently, these pathogenic strains exhibit less than average genetic diversity. Exposure to antibiotics is one selection factor. Kreiswirth et al. (35) suggested that MRSA strains are derived from a very few precursor strains and, in this way, represent a restricted subset of lineages in comparison to the overall diversity among strains within *S. aureus*. Thus, most clinically relevant strains of a given species share virulence factors and/or resistance traits and are often difficult to differentiate from each other.

2. CRITERIA FOR THE EVALUATION OF TYPING SYSTEMS

Several parameters should be considered for evaluating typing systems (3, 41, 64). Adequate efficacy and efficiency are essential characteristics of a successful typing system and the level thereof depends on the epidemiological issue. The efficacy (performance) of a typing system include the typeability, reproducibility, stability, and discriminatory power of a typing system. Typeability refers to the ability of a system to obtain a positive result for each isolate analyzed and is influenced by both technical and biological factors. The technical reproducibility is the ability to assign the same type to a strain tested on independent occasions and testing sites. The biological reproducibility or stability of an epidemiological typing system is the ability of that system to recognize clonal relatedness of strains derived from a common ancestor. Phenotypic or genomic variation may occur during storage or replication of strains in the laboratory (in-vitro stability). Clonal expansion of a strain over a long period of time or during geographically wide-spread outbreaks (in-vivo stability) can also be accompanied by various degrees of genetic variation. A typing system should be able to track this evolution accurately. The discriminatory power refers to the average probability that a typing system will assign different types to two unrelated strains. Ideally, each unrelated strain is identified as unique. In practice a method is useful when the most commonly detected type represents less than 5% of random unrelated strains (29, 30).

The efficiency of typing systems includes several factors, such as the presentation of the epidemiological problem (e.g. local outbreak, population genetics), data processing (analysis, storage), or the financial and technical resources available. Flexibility, rapidity, accessibility and ease of use may be considered as convenience criteria. Flexibility reflects the typeability of a wide range of species with minimal modification of the system. The rapidity of typing techniques ranges from one day to several weeks. Accessibility is primarily based on the cost considerations (reagents, equipment) and the level of technical skill required for the performance of a given method. The ease of use is reflected by the technical simplicity, the workload and the suitability for processing strains and the ease of interpretation of the results. For evaluation of the performance of a typing system, special attention should be paid to the appropriate selection of a bacterial test population. This collection should contain well-characterized strains that includes sufficient members of both epidemiologically unrelated and related isolates.

3. CLASSIFICATION OF TYPING METHODS

A convenient basis for classifying typing systems is to recognize them as phenotypic techniques, those that detect characteristics expressed by microorganisms, and genotypic techniques, those that involve direct analysis of chromosomal or extra-chromosomal genetic elements.

3.1 Phenotypic techniques for *S. aureus*.

Historically, the identification and characterization of microorganisms has been achieved by phenotypic analyses. Phenotypic methods are those that characterize products of gene expression in order to identify strains to the species level and sometimes even to differentiate between strains within a species. Properties such as biochemical pathways, bacteriophage susceptibility, antigens present on the cell's surface, and susceptibility to antibiotics have been used as epidemiological markers. All are examples of phenotypic typing systems that can be applied in the microbiology laboratory. Because these methods rely on gene expression, these tests all have a tendency to vary, based on environmental influences such as changes in growth conditions, growth phase, and the frequency of occurrence of spontaneous mutations.

3.2 Genotyping of *S. aureus* strains.

The advances of molecular biology have provided a variety of new approaches, which have been used for the development of multiple DNA-based strain typing systems. The molecular basis of the different techniques for discriminating individual DNA molecules and the respective targets are summarized in Figure 1.

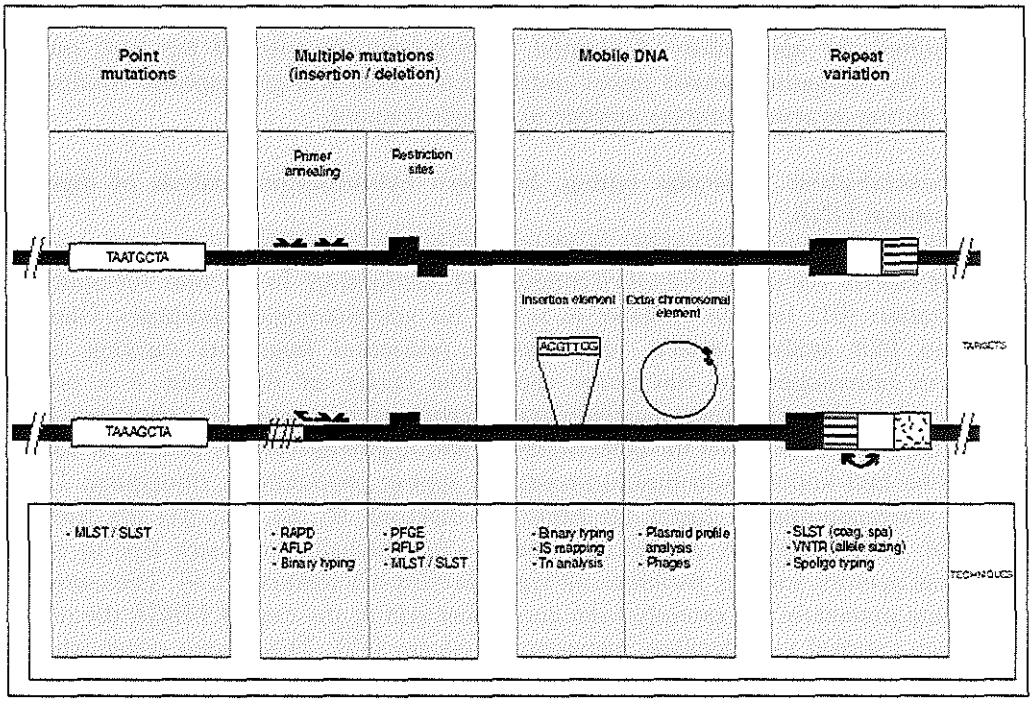

Figure 1. Molecular basis for distinguishing individual DNA molecules: targets and techniques. MLST, multi-locus sequence typing; SLST, single-locus sequence typing; RAPD, randomly amplified polymorphic DNA; AFLP, amplified fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism; IS, insertion sequence; Tn, transposon; VNTR, variable number of tandem repeats.

Over the last two decades DNA-based technologies have been introduced and are increasingly being used in clinical laboratories, which is reflected in the number of papers reporting on *S. aureus* epidemiology, MRSA in particular (73). Over time, several stages of molecular typing methods have found their application in the analysis of *S. aureus* strain collections. These laboratory developments are reviewed chronologically here.

3.2.1 First-phase molecular typing: plasmid profile analysis.

The first DNA-based techniques applied to epidemiological studies of *S. aureus* involved the analysis of plasmids; plasmid analysis was introduced in the mid-70s, (39, 44, 45). Bacterial plasmids are autonomously replicating extra-chromosomal elements, distinct from the chromosome. The analysis of plasmids is a technically simple process. Although plasmids are present in more than 90 percent of MRSA strains, approximately 50 percent of methicillin-sensitive *S. aureus* isolates lack them and can, therefore, not be typed by this approach (8, 24, 25, 72). Also, the reproducibility of plasmid profiling is confounded by structure-variability of the plasmid itself (supercoiled, nicked, linear and oligomeric). This problem can be circumvented by the digestion of the plasmids into restriction fragments and analyzing their numbers and sizes. However, these drawbacks have limited the application of plasmid analysis and the method has only proven effective for evaluating isolates under restricted temporal and geographical conditions, such as during an acute outbreak episode in a single hospital.

3.2.2 Second-phase molecular typing: Southern hybridization analysis of digested chromosomal DNA.

The bacterial chromosome is the most fundamental target molecule in the bacterial cell for the measurement of interspecies relationship. In the mid 70s Southern hybridization (61) became available as a tool for epidemiological purposes. Classical Southern blot analysis detects only specific restriction fragments carrying DNA sequences homologous to the probe used. The choice of the probe is a critical consideration with respect to typeability and discriminatory power and is directly related to the frequency with which the detected restriction fragments vary in number or size, or both. The best-known hybridization-mediated typing procedure is ribotyping. DNA probes corresponding to (parts of) ribosomal operons (2, 23, 67, 81) are used to highlight polymorphism within this operon. All staphylococci carry five to seven ribosomal operons and are, therefore, all typeable with this method (3, 6). The complete ribotyping procedure has recently been automated and in the case of MRSA the results have been coupled to a database management system (85). This library system should facilitate intercenter data exchange and ongoing multicenter studies further explore the possibilities of ribotyping.

A wide variety of DNA probes homologous to mobile genetic elements has been used for the epidemiological analysis of (methicillin-resistant) *S. aureus*. These probes target elements including Tn*554* (35), IS*256* (47) and IS*257*/IS*431* (88). In addition,

different virulence genes such as those encoding coagulase (21, 27, 55), and protein A (17, 58) have been used as targets for DNA probing. Genes encoding virulence factors are typically present only as a single copy on the genome; consequently, proper strain discrimination often requires probing for multiple different genetic loci (33, 62, 69). The use of multiple probes, including virulence factors (26, 46, 59, 60) or antibiotic resistance gene sequences (34, 46) in individual hybridization reactions generates enhanced resolution. Repetitive elements on the staphylococcal genome may expand the specificity and accuracy of strain analysis (10, 83). Finally, the methicillin-resistance encoding *mec* region provides a number of possibilities for deducing DNA probes and accessing genetic polymorphism (35). Application of PCR multiplexing or micro-array techniques using different probes, may facilitate epidemiological studies in the future.

3.2.3 Third-phase molecular typing: PCR-based techniques and pulsed-field gel electrophoresis.

3.2.3.1 PCR-based typing systems.

The polymerase chain reaction (PCR) was developed in the mid-80s and can now be considered one of the major biological technical innovations of the 20th century. The essential property of PCR is the ability to exponentially replicate (amplify) parts of a given template genome, leading to the accumulation of huge number of copies (amplicon) of the original nucleic acid fragment. Several different approaches have been proposed.

Restriction digestion of PCR products. The amplicons of PCR can be digested with specific DNA-splitting enzymes, called restriction endonuclease(s). The amplicon generally contains restriction sites for several of these enzymes. The DNA fragment length between the restriction sites is variable. Restriction fragment length polymorphism (RFLP) is analyzed by gel electrophoresis. DNA polymorphism within the coagulase gene or the rRNA operon of *S. aureus* genomes are targets for RFLP analysis (27, 55). Like most of the staphylococcal surface proteins, sharing common structural features, the extracellular part of the *S. aureus* coagulase displays a high level of variability, based on the presence of a variable number of tandem repeats. The repeat sequences of the coagulase gene are similar and differ in the presence or absence of a *AluI* restriction site. The discrimination of strains with this technique is moderate (21, 69). The resolution can be improved by increasing the number of loci analyzed, or by increasing the number of restriction enzymes per locus analyzed (7). This approach is analogous to multilocus enzyme electrophoresis (MLEE) and the results are suitable for population genetic studies. The rRNA operon displays extensive size and sequence variation in the intergenic spacer region between the 16S and 23S rRNA genes (36). Polymorphism in the ribosomal operons, as defined by for instance ribotyping, can also be identified through PCR-mediated procedures (50, 67).

PCR based on repetitive chromosomal sequences. Short extragenic repetitive sequences, originally identified in *Enterobacteriaceae* can be used as templates (primers) for PCR (79, 87). These repetitive sequences can be found in most bacteria and are scattered around the bacterial genome. These elements are used as PCR targets by using

primers annealing at bordering sequences. These can give rise to PCR product called an inter-repeat fragment. Several studies using primers that target such repetitive sequences have demonstrated only a moderate resolution of this typing method among MRSA strains (11, 65, 74).

Another sort of repetitive sequence analysis by PCR is that of highly polymorphic short-sequence DNA repeats in prokaryotic genomes (76). In *S. aureus*, genes that encode some of the surface proteins that recognize adhesive matrix molecules such as the protein A or the coagulase genes, contain a variable number of contiguous repetitive DNA elements. The bordering sequences of these direct-repeat sequences can form a template for PCR primers. The size variation of the amplicon, reflects the number of direct-repeats units, can be established by agarose gel electrophoresis (17, 21).

Arbitrarily primed PCR. Arbitrarily primed PCR (AP-PCR) was first described in the early nineties (84, 86) and appeared to be discriminatory for MRSA strains (74). The discrimination level obtained with AP-PCR, also known as randomly amplified polymorphic DNA analysis (RAPD) is based on short primers (10 bp). These oligo's are used under low stringency of amplification conditions. The genetic organization of the *S. aureus* genome among different lineages is not identical and is reflected by the various size and numbers of amplified fragments. The inter-laboratory reproducibility is moderate (75). Standardization with help of sophisticated equipment will optimize the reproducibility of this technique (49). PCR fingerprinting provides a generally applicable typing procedure for *ad hoc* epidemiological diagnostics and complies with the convenience criteria.

Amplified fragment length polymorphism analysis. In the mid-nineties, amplified fragment length polymorphism analysis (AFLP) was designed as a typing tool for microorganisms (80, 89). AFLP analysis belongs to the category of selective restriction fragment amplification techniques, which are based on ligation of adapters, i.e. linkers and indexers, to genomic restriction fragments followed by a PCR-based amplification with adapter-specific primers. In a recent study, fluorescent AFLP (fAFLP) is demonstrated to be of high discriminatory value among epidemic MRSA strains (22). This technique reveals complex banding patterns (40 – 75 fragments). However, interpretation criteria (28) and intercenter validation of the reproducibility of AFLP for MRSA are urgently needed.

3.2.3.2 Pulsed-field gel electrophoresis.

Restriction endonucleases that recognize only a few specific sites in bacterial genomes have been described in the late 70s (70). Consequently exposure of DNA to those enzymes yielded large fragments of DNA, called macrorestriction fragments. Initially, these fragments were too large to be separated by conventional agarose gel electrophoresis. However, in 1984 this problem was solved with the introduction of pulsed-field gel electrophoresis (PFGE) by Schwartz and Cantor (54). During PFGE procedure, the orientation of the electric field across the gel is changed periodically. The separation of the DNA fragments by PFGE is primarily based on the time needed by the DNA molecules to reorient themselves in this gel, rather than the speed by which they can migrate in it. PFGE has often been applied for the comparison of bacterial genomes,

sometimes combined with the use of probes (1, 20, 51), and PFGE is now generally accepted as the current "gold standard" for typing MRSA as well as many other bacterial species(37, 38, 50, 52). However, PFGE generates complex banding patterns for which interpretation, internationally accepted guidelines were drawn up and agreed upon (9, 71). Nevertheless, care has to be taken and relatively well-accepted agreements on the optimization of a standard procedure have to be conceived (48), for the intercenter reproducibility of PFGE remains moderate (77).

3.2.3 Fourth-phase molecular typing: sequence typing.

As established previously, the chromosome is the basic target molecule for the characterization of bacteria. Thus, comparison of nucleic acid sequences within the chromosome is the most stringent method by which potential relatedness among strains can be defined. However, sequencing of whole genomes is not feasible when studying large collections of strains within a species. The challenge for sequence-based typing, therefore is to identify region(s) within the genome which exhibit variable and conserved sequences that can be sequenced efficiently. An elegant strategy has been the classification of bacterial isolates on the basis of sequences of internal fragments of six or seven so-called housekeeping genes (40). House-keeping genes are those genes that encode the many proteins that are essential for cell viability. For each gene fragment, the different sequences are assigned to distinct allele identification numbers and the combination of the numbers defined for all gene fragments generates the sequence type (ST). Isolates with the same allelic profile can be considered clonal with a high degree of accuracy. Such typing is called multi-locus sequence typing (MLST) (14, 40, 63). MLST data can be stored in a computer and comparison of results between different laboratories is possible via the Internet (63). MLST has already been developed for the identification of virulent clones of *Neisseria meningitidis* (40), and *Streptococcus pneumoniae* (13, 15, 63). A new development was the establishment of a MRSA database. Despite of the limited age of MRSA, significant allelic polymorphism was defined and the STs correspond with PFGE profiles (12). This suggests that MLST is a highly discriminatory typing method for the identification of MRSA lineages. MLST is thought to be technically very demanding and the technique is suited more to investigate the bacterial phylogeny and evolution of population lineages than for typing many strains in outbreaks and epidemics.

4. COMPARATIVE ANALYSIS OF TYPING METHODS

Unfortunately, for the characterization of S. aureus strains no universally accepted genetic typing system is available at the moment (73). Over the years, many studies in which multiple laboratory techniques were compared and evaluated in a single laboratory by screening of large collections of *S. aureus* strains have been published (18, 31, 36, 52, 53, 55, 69, 78, 82, 88). Various multi-center studies have been published describing problems surrounding the performance of such technique (75, 77) or interpretation of the results (9, 71) of a given typing system. There remains no

consensus as to the optimal procedure for typing staphylococci. Essentially, most of the image-based techniques generate complex banding patterns which are difficult to interpret and they lack interlaboratory reproducibility. The analysis of the results are subjective and for reasons mentioned it is not possible to construct databases that can freely be exchanged internationally. This indicates that, as yet, there is no typing system that is suitable for the establishment of networks and it is foreseen that all procedures that generate fingerprints will in the end be displaced by procedures that produce a binary output (73). The advantage of these latter approaches is simplification of database management, comparison of results and intercenter in-silico data exchange via computer network systems.

5. FUTURE DEVELOPMENTS AND SCOPE OF THE THESIS

Procedures that are based on the generation of DNA banding patterns are likely to be replaced by techniques that produce a binary output. The latter approach will involve probe-mediated identification or DNA sequence elucidation.

Unique elements, that have been identified in the staphylococcal genome and have been applied in current typing systems, can be used as targets for the development of typing probes. House-keeping genes (12, 14, 15), repeat elements, bordering sequences and mobile genetic elements including plasmid-specific genes, insertion sequences or transposons may serve this purpose. Highly standardized strategies require a database against which unknown genotypes can be compared and subsequently assigned to this database as a new type. This approach should be further developed through novel technologies (66).

This thesis describes the development, application and validation of strain-differentiating DNA probes for the characterization of *Staphylococcus aureus* strains in a system, that yields a binary output. By comparing the differential hybridization of these DNA probes to staphylococcal genomes, further insight in the genomic flexibility, the evolutionary clock-speed, the route of transmission, or the possible role of pathogenicity of a given *S. aureus* strain can be obtained.

The generation, and development of the strain-discriminating DNA probes, constituting the binary typing (BT) system, will be introduced in **chapter II**. Chapters III, IV and V provide examples of application of the BT system. The genetic diversity of methicillin-resistant *S. aureus* (MRSA) strains as measured with the BT system is described in **chapter III**. The nationwide spread of a MRSA clone (**chapter IV**), and the epidemiology of bovine *S. aureus* strains (**chapter V**) was monitored with the BT system, and the results were compared with other genotyping approaches. The validation of the binary probes as stable epidemiological markers, described in **chapter VI**, was determined according to generally accepted evaluation parameters. The final technical protocol of the BT system is outlined in **chapter VII**. The feasibility and technical reproducibility of current BT protocol as measured in a multicenter study is presented in

chapter VIII. Finally, **chapter** IX integrates the data presented in the previous chapters and provide future perspectives in the typing of *S.aureus.*

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On the nature and use of randomly amplified DNA from *Staphylococcus aureus*

Chapter

2

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SUMMARY

Various DNA-based methods have been introduced to genetically type *Staphylococcus aureus* strains but not a single technique is universally applicable. In order to search for DNA probes suitable for differentiating strains, randomly amplified polymorphic DNA patterns were generated for 243 *S. aureus* strains and a single isolate of *Staphylococcus intermedius*. All fingerprints were examined for unique amplicons, and the nature of 42 of these DNA fragments was investigated. Partial DNA sequences were determined, and several homologies were discovered with known *S. aureus* sequences (plasmid pSH6 DNA with insertion sequences, *agrA* and *agrB* sequences, *hld* genes, the gene for 23S rRNA, the lysyl tRNA synthetase gene, and the threonyl tRNA synthetase gene) and with genes from other species (*Haemophilus influenzae bexA* gene and *Bacillus subtilis spoF* and *ctrA*). Thirty fragments were of previously unknown origin. In Southern blots containing *Eco*R1-digested DNA from *S. aureus* strains and the *S. intermedius* strain, nine probes demonstrated the capacity to differentiate strains on the basis of the presence or absence of the sequence element in the staphylococcal genome involved. The remainder of the probes displayed restriction fragment length polymorphisms (n=12), hybridized in a homogeneously positive fashion (n=13), or hybridized only with their source-strains (n=8) (four of the latter were specific to *S. intermedius*). Three of the nine strain-specific probes were overlapping, and two of the others were found to display a high level of inconsistency among epidemiologically related strains. Thus, five strain-specific probes remained that, in a 5-digit typing system, accurately distinguished epidemiologically related and unrelated strains of *S. aureus*. We conclude that application of strain-specific DNA probes, selected on the basis of differing randomly amplified polymorphic DNA patterns, promises to become a technically simple, robust, and reproducible tool that may significantly facilitate the study of the epidemiology of *S. aureus* infections.

INTRODUCTION

S. aureus has remained a major cause of nosocomial morbidity and mortality, and methicillin-resistant *S. aureus* (MRSA) emerged in the 1980s as an additional clinical problem (14, 19). Consequently, analysis of the dissemination of MRSA has been a research focus for the past decade. International epidemiologic surveillance requires reliable techniques capable of differentiating independent strains from clonally related strains, and molecular pheno- and genotyping techniques have been optimized for the purpose of studying the spread of MRSA (13, 14, 17). Regretfully, all procedures applied thus far have specific experimental drawbacks. Multilocus enzyme electrophoresis ensures a high degree of typeability but is thought to be technically very demanding and to have limited discriminatory power in clinical epidemiology (19). Pulsed-field gel electrophoresis (PFGE) has proven to be highly discriminatory, and it is suggested to be superior to other genotyping techniques. However, this method is fairly laborious, and the DNA restriction patterns may be difficult to interpret (25). Moreover, interlaboratory standardization of PFGE is still

problematic (5). Randomly amplified polymorphic DNA (RAPD) analysis has proven to be a rapid technique that yields epidemiologically valid results. But, again, its interlaboratory reproducibility needs improvement (27). Thus, the continued need for accurate (geno)-typing systems that can be applied in clinical laboratories is evident.

Since nucleic acid probe technology has already been introduced in clinical diagnostics (6, 29), the application of such probes in epidemiological typing systems should be considered seriously. Gene probes for the study of *S. aureus* epidemiology have been described (15), but these probe-mediated typing systems seem to be rather cumbersome, since they involve Southern blotting, labeling, autoradiography and interpretation of sometimes complex DNA banding patterns. Since it is known that strain-specific DNA probes can be isolated with the aid of RAPD (7, 26), we have set out to obtain and characterize strain-specific DNA probes for *S. aureus*. Figure 1 illustrates the strategy that was followed. When RAPD banding patterns are studied, some of the DNA fragments that are amplified (so-called amplicons) are present in the fingerprints of all strains belonging to a given species (indicated as probe b in Fig. 1). For the medically relevant *Campylobacter* ssp., for instance, some of these fragments could be used to discriminate *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter coli* in an efficient manner (8). On the other hand, some of the amplicons differentiate at the strain-level (indicated as probe c in Fig. 1). The latter DNA fragments arise either as a consequence of PCR primer annealing site variation or as a result of the presence or the absence of the target sequence in the genome of various strains within a species (7, 18). The latter class of amplicons can be used as binary probes: they either do or do not hybridize to a given strain's DNA. With the use of a number of such probes we set out to develop a hybridization technique with a simple plus/minus output, i.e., a binary typing system that may be capable of elucidating relationships among *S. aureus* strains.

MATERIALS AND METHODS

Bacterial strains. Two hundred forty-three strains of *S. aureus* and a single strain of *Staphylococcus intermedius* (ATCC 49052) were identified by standard microbiological methods (12). Strains were obtained from the United States and Portugal (courtesy of F. Tenover, CDC and H. de Lencastre, ITQB, respectively). A large proportion (95%) of the strains were MRSA.

Fifty-nine of these strains had been typed in detail previously (24, 27); 40 of out them were isolated during four well-documented outbreaks and two pseudo-outbreaks. The 19 remaining strains were epidemiologically unlinked U.S. strains. These epidemiologically related and unrelated strains were subdivided into three sets (SA, SB, and SC). The second collection consisted of 184 MRSA strains that were isolated in an epidemiological survey for MRSA prevalence in Portuguese and Spanish hospitals. These strains were all phagetyped and further characterized by RAPD, PFGE and *mecA*/Tn*554* hybridization studies (1).

DNA isolation. All strains were grown overnight at 37°C on brucella bloodagar plates. Five to ten discrete colonies were suspended in 150 µl of 25 mM Tris-HCl (pH 8.0)

- 10 mM EDTA - 50 mM glucose (TEG buffer). To prepare spheroplasts, 75 µl of a lysostaphin solution at a concentration of 100 µg/ml (Sigma Chemical Corporation, St. Louis, Mo.) was added, and this mixture was incubated for 1 h at 37°C. DNA isolation was done according to Boom et al. (4). Briefly, guanidine-hydrothiocyanate was added for cell lysis, and DNA was purified by affinity chromatography with Celite (Janssen Pharmaceuticals, Beerse, Belgium). DNA was eluted from Celite with 100 µl of 10 mM Tris-HCl (pH 8.0) - 1 mM EDTA, and the concentration was estimated by electrophoresis in the presence of ethidium bromide (0.3 µg/ml) (21). Stock-solutions of DNA were adjusted to a concentration of 0.5 ng/µl and stored at -20°C until use.

RAPD analysis. RAPD was performed essentially as described previously (26, 27). Approximately 5 ng of DNA was added per PCR mixture. The mixture consisted of a buffer system containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01 % gelatine, and 0.1 % Triton X-100. Deoxyribonucleotide triphosphates (0.2 mM; Pharmacia Biotech, Uppsala, Sweden) as well as 0.2 U of *Taq* polymerase (SuperTaq; HT Biotechnology, Cambridge, United Kingdom) were present in the reaction mixture. Five different primers and combinations thereof were used in the assays. The codes and sequences of the primers (50 pmol of primer per reaction) were as follows: ERIC-1R, 5'-ATG TAA GCT CCT GGG GAT TCA C-3'; ERIC-2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3'; AP-1, 5'-GGT TGG GTG AGA ATT GCA CG-3'; AP-7, 5'-GTG GAT GCG A-3'; AP-1026, 5'-TAC ATT CGA GGA CCC CTA AGT G-3' (26, 27, 30). The PCR mixture was overlaid with 100 µl of mineral oil to prevent evaporation. Amplification of DNA fragments was performed in a Biomed thermocycler (model 60; Biomed, Theres, Germany) with predenaturation at 94°C for 4 min, followed by 40 cycles of 1 min at 94°C, 1 min at 25°C, and 2 min at 74°C. Amplicons were analyzed by agarose gel electrophoresis containing 1% agarose (Hispanagar; Sphaero Q, Leiden, The Netherlands) in 0.5x Tris-Borate-EDTA (TBE) (21) in the presence of ethidium bromide (0.3 µg/ml) at a constant current of 100 mA during 3 h. After photography (high-speed sheet film 57; Polaroid), DNA fingerprints were compared visually and unique fragments were selected.

Cloning of amplicons. DNA fragments were excised from the agarose gels. The agarose plugs were melted (15 min at 60°C) in the presence of 1 ml of 4M guanidine-hydrothiocyanate - 50 mM Tris-HCl (pH 6.4) - 3 mM EDTA - 1% (wt/wt) Triton X-100, and DNA was further purified according to the method of Boom et al. (4); this was followed by ethanol precipitation. The lengths and concentrations of the DNA fragments were determined by agarose gel electrophoresis in comparison with molecular length markers and known amounts of λDNA, respectively. Ligation of the PCR product into the pGEM-T Vector (pGEM-T Vector System; Promega Corporation, Madison, Wis.) was done by using T4 DNA ligase according to the manufacturer's instructions. Ligated PCR product-pGEM-T vector was transformed to *E. coli* JM 109, and screening was based on ampicillin resistance (100 µg/ml) and β-galactosidase activity (0.5 mM isopropyl-β-D-thiogalactopyranoside [IPTG] and 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal]). White colonies, possibly containing inserts, were inoculated on a second agar to obtain a pure culture.

TABLE 1. Summary of cloned, RAPD-generated, *S. aureus* DNA fragments: probe characteristics

Strain collections and code	Out[a]	Meth[b]	RAPD		primer	clone	hybridization[c]	RFLP (n[d])	strain specificity[e]
			Fragment code	Size (in bp)					
U.S. strains									
SA-01	NH1	R	1a	1600	ERIC-2	A	+	1	15
SA-01	NH1	R	1c	1150	ERIC-2	B	+	3	15
SA-01	NH1	R	1d	1050	ERIC-2	C	+	2	15
SA-04	NO	S	4a	600	ERIC-2	D	+	1	15
SA-08	NO	R	8a	1200	ERIC-2	E	±	1	13
SA-16[f]	NO	S	16a	1200	ERIC-2	F	+	1	16
SB-08	NO	S	28b	1200	ERIC-2	G	+	2	15
SB-11	I	S	31a	1200	ERIC-2	H	+	1	15
SA-08	NO	R	8b	1100	AP-1	I	+	1	15
SA-10	NH2	R	10a	1200	AP-1	J	+	1	15
SB-02	II	S	22a	150	AP-1	K	-	-	-
SC-01	III	R	41a	600	AP-1	L	+	2	15
SA-01	NH1	R	1e	600	AP-7	M	±	1	8
SA-04	NO	S	4a	900	AP-7	N	+	3	15
SA-04	NO	S	4b	400/500	AP-7	O	+	1	15
SA-16[f]	NO	S	16a	900	AP-7	P	-	1	1
SA-16[f]	NO	S	16b	450	AP-7	Q	-	1	1
SA-16[f]	NO	S	16c	400	AP-7	R	-	1	1
SA-06	NO	I	6a	550	ERIC1R	a	±	1	7
SA-20	NO	R	20a	1300	ERIC1R	b	-	-	-
SC-02	IV	S	42a	2500	ERIC1R	c	+	2	15
SA-02	NH2	R	2a	700	1026	d	±	2	6
SA-02	NH2	R	2b	500	1026	e	±	2	6
SA-13	NH1	R	13a	700	1026	f	±	2	6
SA-02	NH2	R	2a	800	AP-1/7	g	+	2	15
SA-02	NH2	R	2b	550	AP-1/7	h	+	1	15
SB-15	I	R	35a	900	AP-1/7	i	±	2	4
SB-15	I	R	35b	800	AP-1/7	j	+	2	15
SB-15	I	R	35c	600	AP-1/7	k	+	1	15
SB-15	I	R	35a	350	E2/AP-7	l	+	2	15
SA-08	NO	R	8a	100	E2/AP-1	n	+	1	15
SC-02	IV	S	42a	450	E2/AP-1	o	-	-	-
Portuguese strains									
HPV 107	ND	R	107b	550	ERIC1R	2B	±	1	8
HDG 117	ND	R	117a	400	ERIC1R	4A	-	-	-
HPV 107	ND	R	107a1	1000	ERIC-2	6C	+	5	15
HPV 107	ND	R	107a2	800	ERIC-2	6K	+	4	15
HPV 107	ND	R	107b1	600	ERIC-2	7B	+	1	15
HPV 107	ND	R	107b2	300	ERIC-2	7E	-	-	-
HPV 76	ND	R	76a	400	AP-7	15A	+	1	15
HPV 107	ND	R	107a	1200	AP-7	16A	+	1	15
HPV 107	ND	R	107b1	400	AP-7	17A	±	1	3
HPV 107	ND	R	107b2	1000	AP-7	17H	+	2	15

[a] Out, epidemiologically related strains from outbreak; NO, unrelated strains; NH, pseudo-outbreak from nursing home; I-IV, outbreaks; ND, not demonstrated.

[b] Meth; methicillin susceptibility test result; S, susceptible; I, intermediate; R, resistant.

[c] +, Homogeneously positive hybridization results; -, uniformly negative hybridization results; ±, strain specific hybridization results with digested (*EcoR*-1) DNA from *S. aureus* strains, selected from the U.S. bacteria collection (24).

[d] N, number of different banding patterns.

[e] Number of strains hybridizing with a particular probe. The probes are tested on a subset of the American collection, consisting of a panel of 15 *S. aureus* strains and 1 *S. intermedius* strain.

[f] *S. intermedius* biotype.

Material from the same colonies was lysed by boiling for 10 min in 100 µl water. Part of this lysate (10 µl) was transferred into a PCR assay for the amplification of insert sequences by the use of SP6- and T7- promotor sequence primers (SP6, 5'-GAT TTA GGT GAG ACT ATA G-3'; T7, 5'-TAA TAC GAC TCA CTA TAG GG-3'). A total of 50 pmol of both primers was used per PCR. The PCR program consisted of a predenaturation step of 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 74°C. The presence and molecular weights of amplified SP6-T7-primed fragments were determined by agarose gel electrophoresis. Amplified inserts were further purified by using the QIAquick PCR spin purification kit (Qiagen, Hilden, Germany). These amplified fragments were used for the generation of DNA probes and for sequencing.

DNA sequencing and homology analysis. The 373 DNA sequencing system (Perkin-Elmer, Foster City, Calif.) was used for sequencing the cloned fragments according to the manufacturer's cycle sequencing protocol by using dye-terminator chemistry (16, 23). Briefly, DNA was amplified in the presence of a thermostable polymerase and primers annealing with the SP6 or T7 sequences. The extended, fluorescently labeled fragments were separated by polyacrylamide gel electrophoresis. Labels were excited by a laser as they passed a detector near the bottom of the gel. The insert sequences were compared with the data from the nucleotide and protein sequence database of the National Center for Biotechnology (Brookhaven Protein Databank, Genbank, EMBL). Sequences were analyzed with the Basic Local Alignment Search Tool (BLAST), a computer model that analyzes the optimal alignment of both the query and subject sequences and measures their similarity, the so-called maximal segment pair (MSP) score (2).

Hybridization studies. Southern blots were prepared as follows: staphylococcal DNA was digested with *Eco*R1 (Boehringer GmbH, Mannheim, Germany) and electrophoresed through 0.8 % agarose gels (Hispanagar; Sphaero Q) in 0.5 x TBE at a constant current of 100 mA for 3 h (21). Gels were pretreated with 0.25 M HCl, and DNA restriction fragments were transferred to a Hybond N$^+$ membrane (Amersham International, Buckinghamshire, United Kingdom) by alkaline, capillary blotting in 0.5 M NaOH and 1.5 M NaCl. Nylon membranes were sealed in plastic bags in the presence of 2 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (21). Probe labeling, hybridization, and detection were performed with the ECL direct labeling and detection systems, according to the manufacturer's protocols (Amersham Life Science, Buckinghamshire,United Kingdom).

The hybridization characteristics of the DNA inserts were defined as follows: initially, a Southern blot containing DNA from 15 epidemiologically independent *S. aureus* strains and one *S. intermedius* strain was used for Southern prescreening. Cloned probes either hybridized with all DNA samples (sometimes showing restriction fragment length polymorphism [RFLP]), did not hybridize at all, or showed hybridization with only a subset of the DNA samples. Subsequently, probes displaying this latter type of differential hybridization were used to screen the entire American collection (24) (see Table 3).

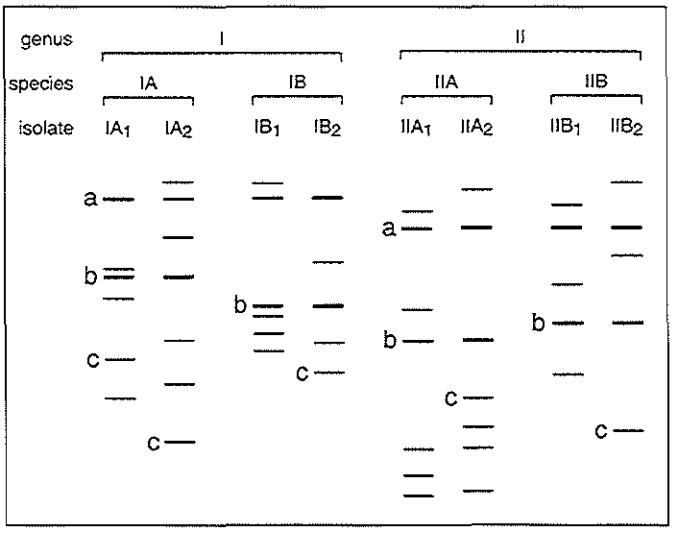

Figure 1. Hypothetical diagram demonstrating the fundamental strategy for the selection of RAPD-generated probes for microbial identification. Probes, suited for the recognition of organisms at different levels (genera, species and strains) can be identified. Prokaryotic DNA was amplified and the DNA amplicons were size-separated by electrophoresis. The lanes represent DNA fingerprints from 8 strains originating from 4 different species belonging to 2 genera. Potential genus-specific (a), species-specific (b) and strain-specific (c) DNA probes are indicated. Strain-specificity of the probes should be verified by Southern hybridization using genomic DNA.

RESULTS

Cloning experiments. Table 1 summarizes all data, comprising three rounds of cloning. In the first experiment RAPD was performed on the 60 U.S. strains. A total of 33 strain-differentiating fragments (Fig. 1, probe c) were selected (ERIC-2, n=13; AP-1, n=10; and AP-7, n=10) from the *S. intermedius* strain (4 fragments) and 11 *S. aureus* strains (29 fragments). Eighteen (55%) fragments were cloned successfully and encoded A to R. The same collection was used in the second cloning attempt. Eighteen fragments (ERIC-1R, n=3; AP-1026, n=4; AP-1 – AP-7, n=7; ERIC-2 - AP-1, n=3; ERIC-2 - AP-7, n=1) were selected from the fingerprints from 8 *S. aureus* strains. In this case, 14 (78%) fragments were clonable and were encoded
a to l, n, and o. In the third experiment, RAPD was performed with the Portuguese *S. aureus* strains. Seventeen unique RAPD fragments from eight strains (ERIC-1R, n=5; ERIC-2, n=5; AP-1, n=4 and AP-7, n=3 fragments) were excised from the agarose gels. Ten of 17 (59%) were clonable. These clones were encoded 2B, 4A, 6C, 6K, 7B, 7E, 15A, 16A, 17A and 17H.

In summary, RAPD genotyping with multiple primers was performed on 243 *S. aureus* strains and 1 strain of *S. intermedius* of U.S. or Portuguese origin. Sixty-eight unique fragments were selected from 26 of the *S. aureus* strains and the single *S. intermedius* strain. Overall, 42 (62%) were successfully cloned.

Sequence data. Sequence data were obtained from both termini (SP6 and T7) of the cloned RAPD fragments. DNA sequences were analyzed separately for homologies with the data in the sequence databank. Most of the nucleotide and protein sequences of the probes did not belong to known DNA elements (71% and 60%, respectively) (Table 2). Probe J appeared to be a part of the *S. aureus* threonyl-tRNA synthetase *thrS*-2 gene, a highly conserved gene as reflected by a long list of (lower-scoring) homologies with other species. Probes d, e, and f showed homologies with *S. aureus* plasmid pSH6 (IS*257* and IS*256* sequences) and Tn*431* and showed similarities with several resistance markers. Probe G had a high score with the *S. aureus* lipase gene and displayed relationship with phage L54 att B (22).

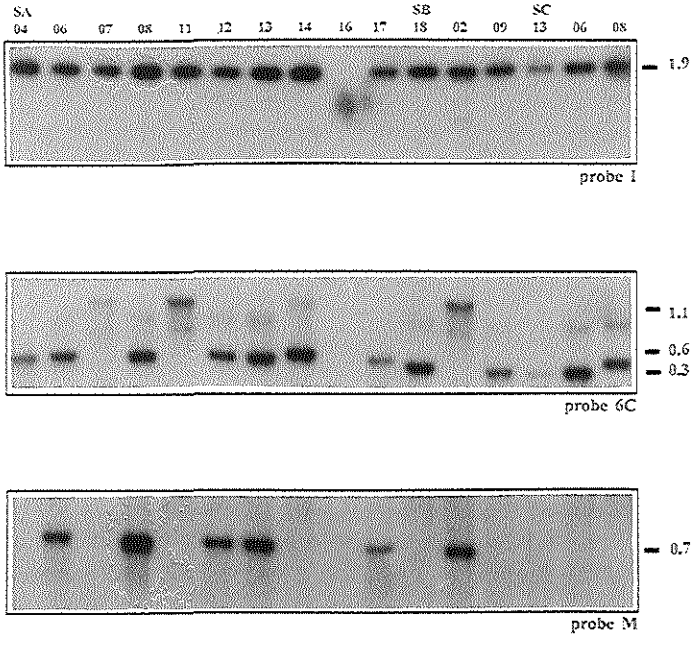

Figure 2. Autoradiographs of *EcoR*-1 digested DNA from 15 *S. aureus* strains and from one strain of *S. intermedius* (SA-16) hybridized with RAPD-derived, labeled probes. Probe I, highly similar with the *S. aureus* gene, which encodes for an energy metabolic enzym (glycolysis), demonstrates uniformly positive results with the *S. aureus* DNA, while 6C, similar with the *S. aureus* 16S rRNA gene, shows RFLPs (4-5 bands). Probe M has strain-specific characteristics. Size is indicated in kilo base pairs on the right.

Note: the strains were derived from the American collection (24), data on strain nature can be found in the latter reference and in Table 3.

TABLE 2. Characteristics of cloned *S. aureus* DNA sequences[a]

	Nucleotide sequence homology		Protein sequence homology	
clone	T7	SP6	T7	SP6
A	*H.influenzae bex A* (327)	none	transport ATP binding protein(130)	none
B	none	none	none	none
C	none	none	membrane protein(321)	membrane protein(321)
D	none	none	none	none
E	none	none	none	none
F	none	none	*traG* (plasm.pSK41)(64)	glycoprotein G (41)
G	none	none	ac.glu.pyrophosphorylase(135)	glutamate receptor (36)
H	none	none	none	none
I	B.subtilis spoF (665)	none	fruc bi-P aldolase(238)	fruc bi-P aldolase (85)
J	Threonyl tRNA synthetase (707)	none	threonyl tRNA synthetase (412)	thr tRNA synthetase (238)
K	none	none	none	none
L	none	none	none	none
M	none	none	none	none
N	none	none	fatty acid ox complex(123)	none
O	none	none	none	none
P	none	none	exonuclease(119)	none
Q	none	none	none	none
R	none	none	p-nitrobenzyl esterase(186)	none
a	none	none	none	*hrmA/glu* transferase(36)
b	none	none	none	none
c	none	none	none	none
d	SA[b] plasm pSH6 (1571)	SA plasm pSH6 (1571)	none	transposase IS257(533)
e	SA plasm pSH6 (376)/IS431	SA plasm pSH6 (693)	none	transposase IS257(241)
f	SA plasm pSH6 (468)/IS431	SA plasm pSH6 (1324)	transposase IS257(444)	none
g	SA lipase (1139)	none	none	none
h	SA *agr A, agrB, hld* (1093)	SA *agr A, agrB, hld* (1714)	*Slug agr*(119)/*SA agrA, B, hld*(321)	*Slug agr/SA agrA, B*(580)
i	none	none	none	phage tail protein (126)
j	none	none	none	none
k	SA *agr A, agrB, hld* (1197)	SA *agr A, agrB, hld* (1197)	*Slug agr*(120)/*SA agrA, B, hld*(373)	*Slug agr/SA agrA, B*(568)
l	none	none	hydr.acyl coA dehydr.(96)	hydr ac coA deh(90)
n	none	none	methionine synthetase(99)	methionine synth. (112)
o	none	none	none	none
2B	none	none	none	none
4A	none	none	none	none
6C	SA 23S rRNA (881)	SA 23S rRNA (1191)	none	none
6K	SA 23S rRNA (598)	SA 23S rRNA (459)	none	none
7B	none	none	none	none
7E	none	none	none	none
15A	B. subtilis gca (447)	none	amino transferase(228)	amino transferase(150)
16A	none	none	*Bsub* prot unknown function(288)	none
17A	none	none	none	lysostaph precursor (101)
17H	none	none	none	none

[a] Cloned DNA sequences of both insert termini, analyzed for similarity on the data of the National Center for Biotechnology Information (NCBI) nucleotide and protein databank with the Basic Local Alignment Search Tool (BLAST) computer program (20. Scores of Maximal Segment Pair are given between brackets. The BLAST program for protein homology compares the 6 reading frame translated products of a nucleotide query sequence (both strands) with the protein sequence database.

[b] SA, *Staphylococcus aureus*

[c] Slug, *Staphylococcus lugdunensis*

Hybridization studies (prescreening). In all pilot hybridization studies, the same Southern blot consisting of *Eco*R1 digests of DNA isolated from 15 epidemiologically unlinked strains and the *S. intermedius* strain, was used (Fig. 2). The 18 cloned probes (A to R) from the first cloning experiment were labeled and hybridized. Four probes, three of which were derived from *S. intermedius*, failed to hybridize to *S. aureus* DNA (see below). However, the probes from *S. intermedius* did hybridize with the *Eco*R1 digest of the homologous DNA. Twelve probes revealed uniformly positive results with *S. aureus* DNA. Probe F obtained from *S. intermedius* (SA-16) displayed hybridization with all staphylococcal DNA digests. Five of 18 probes showed RFLPs (2 or 3 bands). Only two probes (E and M) seemed to be strain-specific and hybridized with DNA from 13and 8 of the *S. aureus* strains, respectively.

The second cloning experiment generated 14 clones (a to o). Two probes showed no hybridization with the *S. aureus* DNA preparation. Seven probes were uniformly positive, four of which showed RFLPs (2 bands). Five probes could be considered strain specific, four of which displayed RFLPs (2 bands). Because of sequence homologies, it was concluded that three of these latter probes (d, e, and f) were at least partially overlapping. Hybridization with these three probes generated concordant results (6 strains of 16). Only 4 of 16 strains hybridized with probe i. The remaining probe, a, hybridized with 7 of 16 *S. aureus* DNA digests.

Hybridization results of the 10 clones from the third cloning experiment (2A to 17H) were as follows: two probes exhibited no hybridization (see below); six probes hybridized uniformly (excluding *S. intermedius*), of which three showed RFLPs (2 to 5 bands); and the remaining two probes displayed strain-specific properties (2B and 17A).

Probes K, b, o, 4A and 7E did not hybridize with any of the staphylococcal DNA tested. In order to demonstrate whether staphylococcal DNA was cloned, the probes were tested to their source strains; SA-20, SC-02, HDG 117 and HPV 107, respectively and displayed positive results. As a result of the stringent conditions, combined with the small size of this probe, no hybridization-signal was obtained with source strain SB-02. In summary, 42 cloned staphylococcal RAPD fragments were screened for hybridization to *Eco*R1 digests of DNA from a subset of 15 *S. aureus* strains. Eight probes hybridized with their parent strain only, 25 reacted uniformly with all strains (many of which displayed RFLPs [1 to 5 bands]), and 9 probes showed some degree of strain specificity. However, three of these strain-specific probes appeared to be overlapping. Thus seven probes (E, M, a, d, i, 2B, and 17A) were finally identified and were included in the further epidemiological evaluation.

Epidemiology and validation. The entire U.S. collection of staphylococci (24) was typed by hybridization with the seven strain-specific probes (Table 3). Upon epidemiological clustering of the strains, it became apparent that probes i and 2B displayed a low level of reproducibility among epidemiologically and genetically related strains. In further validation of the genotyping with strain-specific probes, so-called binary typing, these two probes were excluded from the overall analysis which is presented in Table 3. If epidemiologically unlinked strains were analyzed, the five strain-specific probes could discriminate 12 genotypes in a group of eighteen strains. *S. intermedius* DNA was not recognized by any of

the probes. Interestingly, the overall combined consensus probe type III (Table 3) encompassed 80 % of all MRSA strains. The maximum incidence of a single probe type in the epidemiologically unrelated group was 5 of 18 (28 %). All the epidemiologically related clusters of strains showed that the incidence of a single type was always >75 % (Table 3). Many of the *S. aureus* strains that had been cultured during an outbreak but had shown epidemiologically or genotypically aberrant characteristics, were also different when analyzed by our probe-based binary typing system (e.g. strains SA-14, SB-11, SC-11, and SC-15; Table 3).

DISCUSSION

Development of a novel binary typing system for *S. aureus*. The use of PCR for amplification of ribosomal sequences and deduction of specific ribosomal probes provides an excellent example of how DNA-based diagnostics can be used to define species nature (9). Theoretically, similar probe-based techniques can also be used to distinguish among strains within a species. In the present communication we describe the preliminary phase in the development of a new binary typing system for *S. aureus*, based on a simple, binary output from DNA probes that display strain specificity. This PCR-mediated approach is based upon the development of DNA probes that are selected by RAPD genome scanning (Fig. 1). Several examples of the identification of genus- and species-specific probes by means of RAPD have already been presented (7). In two of these instances, a probe capable of differentiating among strains of a given species has been proposed (7, 26). Another recent example is provided by the development of a *Staphylococcus pasteuri*-specific DNA probe, also derived from a RAPD fragment (29). Via RAPD analysis, we tried to identify DNA regions in the *S. aureus* genome that enable the isolation of strain-specific DNA probes. This type of discriminatory DNA probes hybridize in a plus/minus fashion with complete genomic DNA molecules from different strains. RAPD fragments were amplified in only a subset of *S. aureus* DNA preparations were selected. Hybridization studies subsequently revealed that only approximately 15% of these fragments displayed the desired molecular typing characteristics. This implies that appearance of most of the other RAPD fragments were the result of annealing site mutation or minor insertional and/or deletional events.

The nature of differentially amplified DNA in *S. aureus*. In several cases where homology with known DNA motifs was observed, previous data exists that show these DNA sequences to be flexible (Table 2). The nature of the DNA probes with strain-specific characteristics used in this study may be homologous to mobile genetic elements, although only two probes (d and i) represent genes previously known to be related to such elements. These are the transposase encoded by IS*257* and a phage tail protein, respectively. The molecular basis of the variation in patterns of genotyping systems, such as insertion sequence typing using IS*431* (3) or hybridization with probes targeting genes or sequence elements [e.g., *agr*, *mec*, Tn*554*, *aph*(2")-*aac*(6')] (15), are all related to mobile genetic elements. Some of these are found by RAPD-based selection as well.

TABLE 3. Epidemiology, phenotype and genotype of 60 epidemiologically related and unrelated strains of *S. aureus*, compared with their hybridization patterns obtained with 5 strain-specific RAPD generated probes [a]

Cluster[c]	Strain code	Methicillin susceptibility[b]	Phage type[c]	IS type[d]	RAPD type[e]	Ribo type[f]	PFGE type[g]	MLEE type	RFLP type[h]	Hybridization pattern with probe: i[i]	2B[i]	E	M	a	d	17A	Hybridization type[j]	Identity value (%) [probe type[k]]
non related	SA-4	S	6/47/54/75	NH	BBB	fi	E	E	NH:X:4:NH	-	-	+	+	+	-	+	I	
	SC-3	S	6/47/54/75	NH	BJB	ai	C	B	NH:NH:4:NH	+	+	+	+	+	-	+	I	11 (I)
	SA-6	I	NR	B	AAC	aa	C	A4	II:NH:1:a	-	+	+	-	+	+	+	II	6 (II)
	SA-7	S	53/+	NH	AAC	bc	B	A2	NH:NH:1:NH	-	-	+	+	+	+	+	III	
	SA-8	R	54/75/77/81	D	CCD	ed	G	D1	I:NH:6:NH	-	+	+	+	+	+	+	III	
	SA-12	R	47/54/75/77/83A	C	AAA	bb	J	A5	I:A:1:NH	-	+	+	+	+	+	+	III	
	SB-14	S	54/75/77	D	EDE	ci	H	C	NH:NH:1:NH	+	+	+	+	+	+	+	III	
	SB-18	R	75/+	E1	AAA	aa	A	A1	I:A:1:a	+	+	+	+	+	+	+	III	28 (III)
	SA-11	R	NR	G	CCD	gd	F	D2	II:NH:6:NH	-	+	+	-	-	+	-	IV	6 (IV)
	SA-16[b]	S	NR	NH	FEF	de	I	F	NH:NH:NH:NH	-	-	-	-	-	-	-		
	SA-18	R	47/54/75/77/83A	C	AAA	bb	J	A3	I:A:1:NH	-	+	-	-	-	-	-	V	6(V)
	SA-20	R	47/54/75/77/83A	C	AAA	bb	J	A1	I:A:1:NH	-	+	-	+	+	-	+	VI	6 (VI)
	SB-1	R	75/77	E	AAA	aa	A1	A1	I:Y:1:a	-	-	-	+	+	+	+	VII	6 (VII)
	SB-7	S	6/47/54/75	NH	BBB	ci	D	B3	NH:X:4:NH	-	+	-	+	-	-	-	VIII	6 (VIII)
	SB-8	S	95	NH	HBI	dd1	F	C	NH:NH:1:NH	+	+	+	-	-	-	-	IX	
	SC-8	S	NR	NH	HKI	b1g	B1	A3	NH:NH:1:NH	-	+	+	-	-	-	-	IX	11 (IX)
	SB-9	S	3A	NH	IDJ	bb	B	B1	NH:Z:7:NH	+	+	+	+	+	+	-	X	
	SB-13	S	3A	NH	GGJ	bb	B1	B2	NH:NH:7:NH	-	-	+	+	+	+	-	X	11 (X)
	SB-16	R	75/77/83A	E	AAA	aa	A1	A1	I:Y:1:a	-	-	+	-	-	-	+	XI	6 (XI)
	SB-17	I	96	NH	JIM	fj	E	A2	NH:NH:1:NH								ND	
pseudo-outbreaks																		
NH-1	SA-1	R	54/77	A	AAA	aa1	K1	A1	I:A:5:a	-	-	+	+	+	+	+	III	
	SA-3	R	47/54/75/77	C	AAA	aa	A	A1	I:A:1:NH	-	+	+	+	+	+	+	III	
	SA-9	R	54/77	A	AAA	aa1	K2	A1	I:A:5:a	-	+	+	+	+	+	+	III	
	SA-13	R	54/77	A	AAA	aa	A	A2	I:A:1:a	-	+	+	+	+	+	+	III	
	SA-19	R	54/77	A	AAA	aa1	K3	A1	I:A:1:a	-	+	+	+	+	+	+	III	83 (III)
	SA-14	S	54/75/77	NH	EDE	ci	H	C	NH:NH:1:NH	+	+	+	-	-	+	-	IV	17 (IV)
NH-2	SA-5	R	77	A	AAA	aa	A	A1	I:A:1:a	-	-	+	+	+	+	+	III	
	SA-10	R	77	A	DAA	aa	D	B	I:A:1:a	-	-	+	+	+	+	+	III	
	SA-15	R	77	A1	AAA	aa	A	A5	I:A:1:a	-	-	+	+	+	+	+	III	
	SA-17	R	54/75/77	A	AAA	aa	A	A1	I:A:1:a	+	+	+	+	+	+	+	III	100 (III)

table to be continued on next page

	SA-2	R	75/77	A1	AAA	aa	A	A1	I:A:1:b								ND	
outbreaks																		
I	SB-3	R	75/+	E	AAA	aa	A	A1	I:A:1:a	+	+	+	+	+	+	+	III	
	SB-5	R	75/+	E	AAA	aa	A	A1	I:A:1:a	+	+	+	+	+	+	+	III	
	SB-10	R	75/+	E	AAA	aa	A	A1	I:A:1:a	+	-	+	+	+	+	+	III	
	SB-15	R	75/77/83	E	AAA	aa	A	A1	I:A:1:a	+	-	+	+	+	+	+	III	
	SB-19	R	75/+	E	AAA	aa	A	A1	I:A:1:a	+	-	+	+	+	+	+	III	
	SB-20	R	75/+	E	AAA	aa	A	A1	I:A:1:a	+	+	+	+	+	+	+	III	86 (III)
	SB-12	R	75/+	E	AAA	aa	A1	A1	I:A:1:a	+	-	+	-	-	-	+	XI	14 (XI)
II	SB-2	S	3A/55	NH	GFH	bb	B	B1	NH:NH:7:NH	+	+	-	-	-	-	-	V	
	SB-6	S	3A/55	NH	GFH	bb	B	B1	NH:NH:7:NH	-	-	-	-	-	-	-	V	
	SB-4	S	3A/55	NH	GFH	bb	B	B1	NH:NH:7:NH	+	+	-	-	-	-	-	V	75 (V)
	SB-11	S	3A/55	NH	GFK	b1b	C	B1	NH:NH:7:NH	+	-	+	+	+	+	+	III	25 (III)
III	SC-1	R	75	F	BJB	ab	A	A1	I:A:4:a	+	-	+	-	+	+	+	II	
	SC-4	R	75	F	BJB	ab	A	A1	I:A:4:a	-	+	+	-	+	+	+	II	
	SC-5	R	NR	F	BJB	ab	A	A1	I:A:4:a	+	-	+	-	+	+	+	II	
	SC-9	R	75	F	BJB	ab	A	A1	I:A:4:a	+	-	+	-	+	+	+	II	
	SC-12	R	75	F	BJB	ab	A	A1	I:A:4:a	-	-	+	-	+	+	+	II	
	SC-14	R	75	F	BJB	ab	A	A2	I:A:4:a	-	-	+	-	+	+	+	II	
	SC-17	R	75	F	BJB	ab	A	A1	I:A:4:a	-	+	+	-	+	+	+	II	
	SC-20	R	75	F	BJB	ab	A	A1	I:A:4:a	-	+	+	-	+	+	+	II	80 (II)
	SC-11	R	75	NH	BJB	ab	A	A1	I:A:4:NH	-	+	+	-	+	-	+	XII	10 (XII)
	SC-15	R	75	F	BJB	b2b	A	A1	I:A:4:a	+	-	+	+	+	+	+	III	10 (III)
IV	SC-2	S	52/52A/80/47/54/ 83A/84/95	NH	HKI	bg	B	C1	NH:NH:1:NH	-	-	+	-	-	-	-	IX	
	SC-7	S	95	NH	HKI	bg	B	C1	NH:NH:1:NH	-	-	+	-	-	-	-	IX	
	SC-13	S	95	NH	HKI	bg	B	C1	NH:NH:1:NH	-	-	+	-	-	-	-	IX	
	SC-16	S	95	NH	HKI	ag	B	D1	NH:NH:1:NH	-	-	+	-	-	-	-	IX	
	SC-18	S	95	NH	HKI	bg	B	C1	NH:NH:1:NH	-	+	+	-	-	-	-	IX	
	SC-19	S	95	NH	HKI	bg	B	D2	NH:NH:1:NH	+	+	+	-	-	-	-	IX	75 (IX)
	SC-6	S	95	NH	HKI	bg	B	C1	NH:NH:1:NH	-	+	+	-	+	+	+	II	12,5(II)
	SC-10	S	52A/79/80/47/54/ 75/77/83A/95	NH	HKI	bg	B	C1	NH:NH:1:NH	+	+	+	+	+	-	+	I	12,5 (I)
Number of types[1]		3	18	9	16	15	18	6	17								12	

table to be continued on next page

[a] Methicillin susceptibility testing, phage type, and PFGE, multi locus enzyme electrophoresis (MLEE), and RFLP typing was performed by Tenover et al (24). RAPD analysis was done by van Belkum et al (26).
[b] S, susceptible; I, intermediate; R, resistant.
[c] NR, nonreactive.
[d] Insertion sequence typing based on RFLP obtained by using the IS*431* sequence as a probe (3).
[e] Three letter code summarizes the typing results per primer used (first digit, primer 1; second digit, primer 7; third digit, primer ERIC-2).
[f] Results obtained with *Hind-III* and *Cla-I* (first and second letters, respectively).
[g] PFGE types were assigned within each of the three sets of 20 isolates (SA, SB, SC).
[h] RFLP, genotyping based on RFLP of *Cla*-1 digested chromosomal DNA using the variable gene probes; *mec*, Tn*554*, *agr* and *aph*(2")-*aac*(6').
[i] Overall result after hybridization with the 5 strain-specific probes; NT, no hybridization; ND, not done.
[j] Percentage occurrence of a given probe type. Non-related, pseudo-outbreaks (NH1, NH2) and outbreak clusters (I to IV) were analyzed separately.
[k] Hypervariable probes.
[l] *S. intermedius* biotype.

The occurrence of members of multigene families in DNA homology searches including the tRNA synthetases is also not surprising. This could be expected on the basis of their frequent occurrence throughout the entire chromosome and in relation to previously documented positional variability of this type of gene family (20). Also, novel and potentially variable elements, such as CTP synthetases, amidotransferases and the *bex* homologs, were identified during our study in *S. aureus*. All these regions, probably differentially amplified because of the sequence variation in primer sites, are potential targets for comparative sequencing studies aiming at the elucidation of the genetic basis of this variability.

Molecular typing of *S. aureus* by differential binary hybridization testing. The usefulness of the current panel of seven strain-specific, RAPD-derived probes was evaluated by using a well-characterized collection of epidemiologically and genetically (un)related *S. aureus* strains (24). None of the probes proved to be exclusively MRSA specific, and both MRSA and methicillin-sensitive *S. aureus* are typeable by all probes. Strains SA-04, SB-07, and SC-03, representing ATCC 12600, did not show corresponding probe data. This finding is supported by the results of the PFGE and RFLP typing analyses used in Tenover et al.'s study (24). This phenomenon is probably due to multiple subcultering and lyophylization of these strains. It was demonstrated that by summarizing hybridization events into a seven-digit typing code, a moderate agreement with epidemiological and typing data previously established by Tenover et al. (24) was obtained (Table 3). However, if the results obtained with probe i and 2B were deleted from the digital codes, the degree of concordance increased significantly. Probe molecules i and 2B cannot be used for epidemiological studies because of the inconsistent presence of homologous sequences among outbreak strains representing a single strain. The precise molecular basis for this "hypervariability" is not yet understood.

The identity values (75 to 100%) of a single probe type among the epidemiologically and genetically related strains in the four outbreak clusters are highly encouraging (Table 3). The overall resolution among the unrelated strains, however, was decreased when compared

with the data described by Tenover et al. for PFGE (24). The maximum incidence of a single probe type has demonstrated to be 28% (5 of 18). In practice, a technique is statistically useful when the most commonly detected type represents less then 5 % of the random unrelated strains (10, 11). To improve the reproducibility and discriminatory power of our typing method, we are currently focusing our efforts on expanding the number of probes and simplifying the hybridization-based test format. This effort may also render typeable the two strains from the present study that were nontypeable (SB-02, SB-04, and SB-06, isolated from one patient, and SA-18 did not hybridize with the probes in the present panel). Also, validation of our typing method with other well-characterized collections of *S. aureus* is being pursued.

Conclusion and further developments. The binary typing procedure described herein may in the long run be preferred over all RFLP typing analyses, including PFGE, because interpretation and standardization of complex banding patterns are not required (25). Moreover, DNA hybridization assays can be performed in simple, non-radioactive 96-well-plate-based test systems, allowing implementation of this assay in most routine microbiology laboratories. It is theoretically also possible to translate the DNA sequences of the selected probes into strain-specific primers for a binary PCR assay (18). This may simplify and speed-up binary typing of *S. aureus*. The binary typing method promises to become a technically simple and reproducible typing system that can be extrapolated to other species (7).

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Genetic diversification of methicillin-resistant *Staphylococcus aureus* as a function of prolonged geographic dissemination and as measured by binary typing and other genotyping methods

Chapter

3

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SUMMARY

The aim of the present study was to determine the extend of genome evolution among methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Three different collections of strains were analyzed, comprising locally, nationally and internationally disseminated genotypes. Various genotyping assays displaying different levels of resolution were applied. Geographically and temporally diverse MRSA comprised the international group. MRSA strains recovered during an outbreak in a New York City hospital and Portuguese MRSA isolates, all resembling the so-called Iberian clone, were included in the local and national collections, respectively. Genotypes were determined by genome scanning typing techniques and procedures which analyze specific DNA elements only. The outbreak strains showed subclonal variation, whereas the Portuguese isolates displayed an increasing number of genotypes. Among the epidemiologically unrelated MRSA strains, the different genotyping techniques revealed a wide heterogeneity of types. Different typing techniques appeared to show different levels of resolution, which could be correlated with the extend of geographic spread; the more pronounced the spread, the higher the degree of genome evolution. Binary typing and randomly amplified polymorphic DNA analysis are the typing methods of choice for determining (non)identity among strains that have a recent common ancestor and have undergone yet limited dissemination.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are reputed to represent a limited number of genetic lineages (15). Evolution of the methicillin resistance defining *mec*A gene and adjacent regions is mainly reflected by deletion events within the regulatory genes *mec*R1 and *mec*I, (3, 7, 8, 18, 22) and multiple horizontal gene transfer events have occurred in the past (15). The genetic background in which the *mec* region DNA is embedded, codetermines the clinical threat posed by a given strain, and frequently, so-called high-level resistant MRSA strains are found (9, 13, 19). The background also determines other clinically significant factors such as epidemicity and virulence (14). Clearly, DNA typing of MRSA is important because the nature of the genome in which the *mec*A gene is located is of pivotal pathogenicity-related importance. Appropriate overall genotyping techniques for MRSA include PFGE (28), RAPD analysis (27), Tn*554* probing (12), multilocus enzyme electrophoresis (MLEE) (15), and binary typing (BT) (29). Other typing strategies identify polymorphism in a single DNA element, such as the *spa* gene (6), *mec*A (12), *mec*-regulator genes (22) or the *cna* gene (20). Various target sequences may accumulate mutations at a different evolutionary clock speed. By using a diversity of typing techniques on well-defined strains of MRSA, these different clock-speeds may be deduced from the experimental data. For this reason, we determined gene and genome diversification among locally, nationwide, and worldwide disseminated MRSA strains,

using various molecular typing procedures.

MATERIALS AND METHODS

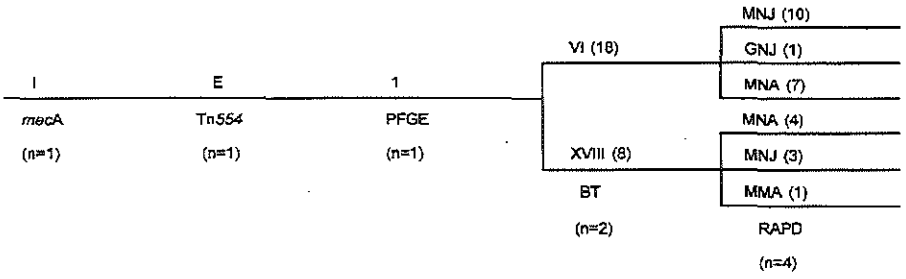
Bacteria. The demographic data of the 103 strains of MRSA used in this study are displayed in table 1. For cultivation, bacteria were inoculated on Columbia agar base (Oxoid, Brunswig, Amsterdam, the Netherlands) supplemented with 5% sheep blood. The plates were incubated at 37°C for 24 h. All strains were identified as *S. aureus* by standard microbiological methods (10). Methicillin resistance was determined by inoculation of the strains on Mueller-Hinton agar (Oxoid CM337; Brunswig, Amsterdam, the Netherlands) in the presence of a disk containing 5 µg of methicillin (Oxoid; Brunswig, Amsterdam, the Netherlands). Zone diameters were interpreted according to the NCCLS guidelines (21).

Table 1. *Demographic data from the MRSA collection.*

MRSA collection			No.	*Source/reference*
* *geographically and temporally diverse strains* (worldwide)			65	(12)
Origin(+)	*Early strains* (60s)	*Recent strains* (80s)		
US	4	35		
Canada	-	10		
Europe	7	6		
Africa	2	-		
(+) *one strain from unknown origin and isolation date.*				
* *Isolates of a local epidemic strain from a* New York City *hospital.*			26	PHRI
* *nationwide epidemic strains from* diverse Portuguese *hospitals,* (Lisboa, Coimbra, Guarda, Gaia, Oporto, Viana Castelo) *representing the* Iberian *clone* (1992 *to* 1994).			12	(1)

PHRI = Public Health Research Institute, New York.

A. local outbreak strain (NYC, n=26)



B. Nationwide epidemic strain (Iberian clone, n=12)

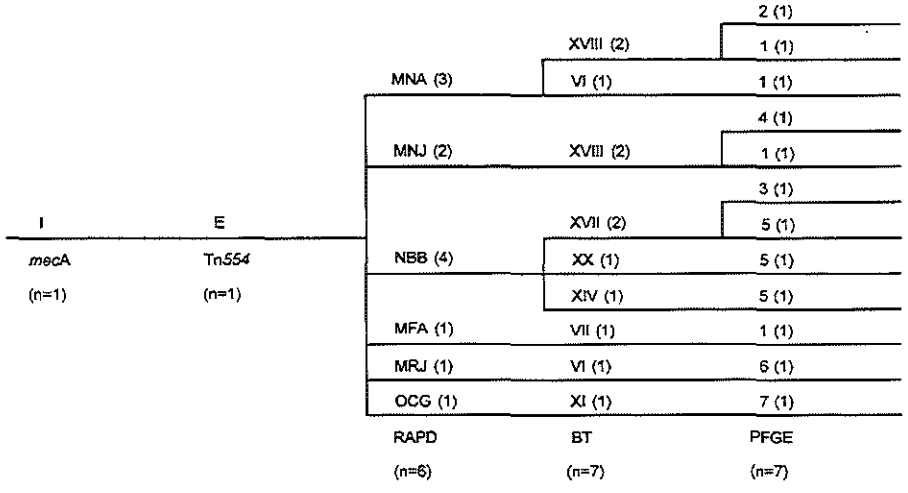
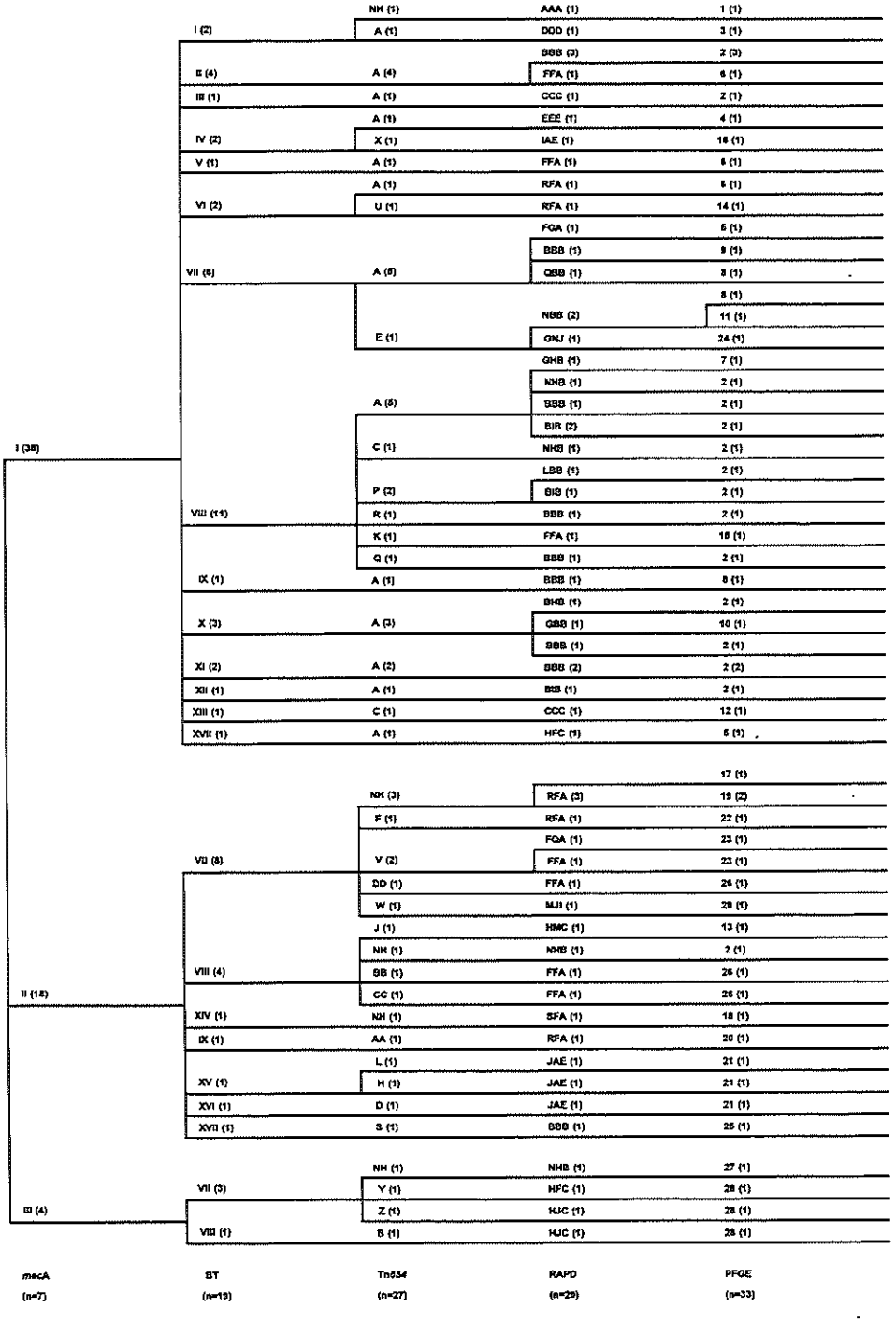


Figure 1. Diagram illustrating the similarities and differences between the genotypically defined groups of MRSA by whole genome typing strategies. The type distributions of the local outbreak strain, the nationally dispersed epidemic strain and the epidemiologically unrelated strains are displayed in figure 1a, 1b and 1c (see next page), respectively.

Note to figure: After the genotype, the number of strains within a distinct genotype is indicated in brackets. The number of different genotypes defined by each typing method is shown in brackets beneath the genotyping procedure used. The typing procedures are arranged according to increasing level of resolution within each MRSA cluster. A total of 6 MRSA strains with *mec*A types IV through VII (detected from figure 1c) were occasionally encountered and could be differentiated with each genotyping system.

C. Worldwide collection of MRSA strains (n=65)



Binary Typing (BT). Binary typing was performed as described by Van Leeuwen *et al* (29). Probe labeling, hybridization and detection were performed with ECL™ (Amersham Life Science, Buckinghamshire, England). Five different DNA probes (E, M, a, d, 17A) were applied. Hybridization was scored with a plus (1) or a minus (0) according to the presence or absence of hybridization signal, respectively (see figure 4). The combination of binary data revealed an overall code depicted as a Roman numeral.

RAPD analysis. RAPD analysis was performed as described previously (27). Banding patterns were compared visually and even a single band difference defined a novel RAPD type. The three-letter codes, based on ERIC2, AP-1, and AP-7 priming (29), are presented in figure 1. Codes determined for the epidemic versus the diverse strains cannot be compared.

PFGE. Restriction with *Sma*-1 (Boehringer Mannheim, Germany) and subsequent separation of the DNA macrorestriction fragments was performed by PFGE as described before (28). Macrorestriction profiles were interpreted as described by Tenover *et al* (24) and each pattern is presented as a numeral.

***mecA*/Tn*554* probe typing.** DNA was digested with *Cla*-1 endonuclease (New England Biolabs) according to the manufacturers' instructions. Generation of target-specific probes and hybridization were done as described previously (12).

***cna* probe.** Presence or absence of the *S. aureus* collagen adhesin (*cna*) was used as an additional genotypic marker for the differentiation of MRSA strains. Probing was performed essentially as described by Smeltzer *et al* (20).

PCR of *mecA* regulator genes, *mecI* and *mecR1* (5'- and 3'- end region). PCR was performed as described previously (22). Three sets of primers were used to detect different regions of the *mec* regulator genes: (1) *mecI*, (2) the 5' end (transmembrane part), and (3) the 3' end (penicillin-binding part) of *mec*R1.

RESULTS

The different groups of strains were analyzed with whole genome and gene typing techniques. For each of these approaches, strains were clustered according to geographical origin. MRSA strains, isolated during a local outbreak, upon nationwide spread or upon worldwide dissemination, were distinguished.

Whole genome typing methods. Analysis of the local New York city cluster revealed that the isolates of the epidemic strain display "subclonal" variation as measured with RAPD analysis and BT (figure 1a) within a conserved genetic background (identical *mec*A/Tn*554*/PFGE pattern). RAPD analysis revealed four different patterns (types MNA, MNJ, MMA, GNJ). However, 3 of these types (MNA, MNJ and MMA) showed a homology of >96%, estimated by Dice coefficient of correlation and by cluster analysis with UPGMA. One RAPD type was drastically different (GNJ) and displayed a homology of only 80% with the former cluster. BT distinguished 2 types, the frequencies of occurrence of which were 69% and 31%,

respectively, and deviated by 2 out of 5 probes.

The isolates of the Iberian clone displayed an increasing number of genotypes (see fig. 1b). PFGE revealed 7 genotypes, among which the most predominant type (1; prevalence 33%) was genetically related to the local outbreak cluster due to a similar *mec*A/Tn*554*/PFGE type. Accordingly, RAPD analysis and BT also displayed more distinct types (6 and 7, respectively). The geographically and temporally diverse strains showed a large number of *mec*A/Tn*554* types (7 and 27, respectively). BT revealed 19 genotypes, whereas Tn*554* probing, RAPD analysis and PFGE showed 27, 29 and 33 different patterns, respectively. Combined typing results differentiated all of the strains into unique genotypes. A survey of the data obtained by different whole genome typing techniques is presented in figure 1c, and examples of PFGE and RAPD patterns are depicted in figure 2.

Gene typing methods. A survey of the data obtained with typing strategies targeting specific DNA elements demonstrated limited variation in the number of polymorphs. The distribution of the *mec* regulatory genes, *mec*I, *mec*R1 (5' and 3' part), is shown in table 2 and figure 3. All ancient European MRSA strains (7/7) from the worldwide collection revealed a deletion event in the *mec* regulatory genes. The prevalence of the latter phenomenon is much lower among the US (7/39, 18%) and Canadian (1/10, 10%) MRSA strains. A large proportion of these US strains (6/7, 86%) lacking the *mec* regulatory genes originated from New York. None of the epidemic strains, local or nationwide, show the presence of complete *mec* regulatory genes. Among the 65 diverse MRSA strains only 5 (9%) harbored the *cna* gene. In the case of the Iberian type strains, only one isolate (2090) contained the gene encoding the collagen adhesin.

DISCUSSION

More than 90% of the clinical MRSA isolates possess the *mec*A locus on their genome (7), which is a highly conserved region (11). Heterogeneity in the degree of resistance (25) can in part be explained by genomic diversity in the *mec* regulator region (7, 16). Clonal spread of MRSA has been suggested (12) and examples of the epidemic behavior of some MRSA strains have been documented in several European countries including Poland (26), Germany (30), Denmark (17), the UK (4), Hungary (5), Turkey (27), and the Iberian peninsula (1).

Also in other continents, epidemic MRSA strains have been described (23). Since clonal relatedness can only be deduced from (molecular) studies comparing isolates from MRSA, and since relatively little is known about the comparative resolution of modern methods, we here compared several well-defined clusters of MRSA strains by a variety of techniques.

Our main research goal was to establish whether or not the degree of geographic spread correlated with the extend of genetic change as detected by different techniques, either in a whole genome or in a more local (gene) fashion.

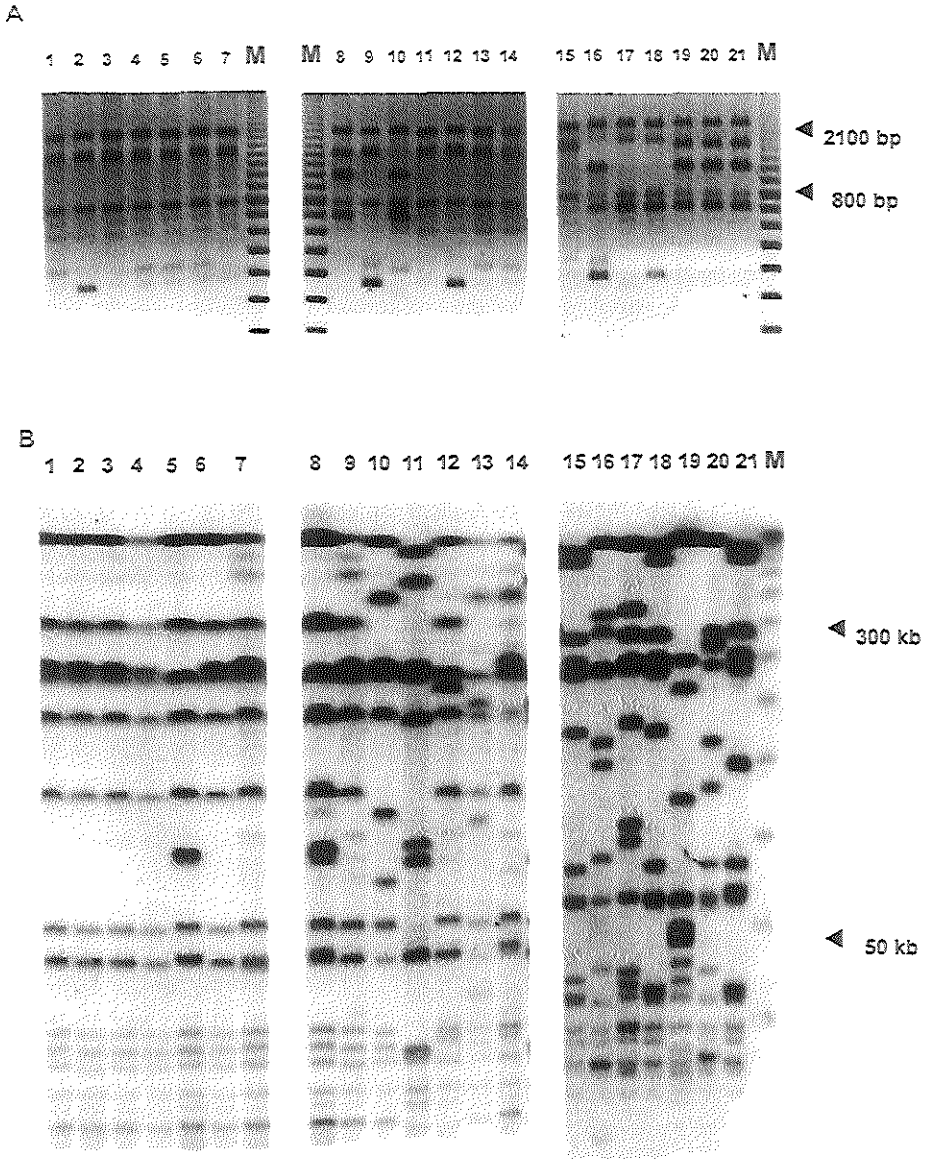


Figure 2.Representative examples of the results of (A) PCR-mediated DNA fingerprinting using primer AP7 and (B) PFGE patterns after DNA digestion with *Sma*I.
Isolate numbers are indicated above the lanes and represent the local outbreak in a NYC hospital (1 to 7), the Iberian clone (8 to 14) and the worldwide MRSA strains (15-21). M lanes contain molecular length markers, sizes of which are indicated on the right.

Since genetic instability depends on DNA composition or structure, detailed studies on its influence on the outcome of genetic typing are urgently required.

Table 2. *Analysis of mecI, mecR1 (5') and mecR1 (3') polymorphs within the total MRSA collection.*

MRSA *collection*		Distribution of *mec regulator genes* I, R1(*5'*), R1(*3'*) + + +	I, R1(*5'*), R1(*3'*) - + -
Iberian *clone* (*n*=26)		0	26
Local outbreak strain (*n*=12)		0	12
non-related MRSA *strains* (*n*=65)[§]			
* US	(60-80)	*3*	1
	(>80*s*)	2	96
* *Canada*	(60*s*)	0	0
	(>80*s*)	9	1
* *Europe*	(60*s*)	0	7
	(>80*s*)	*4*	2
* Africa	(60*s*)	0	2
	(>80*s*)	0	0

[§]) *one strain from unknown origin and isolation date.*

Short-term dissemination. The outbreak of MRSA infections in a local New York City hospital provides a cluster of strains forming the basis for the analysis of short-term genome evolution. The outbreak was defined by *mec*A/Tn*554*/PFGE typing (type I/E/1). RAPD analysis and BT defined "subclonal" variation. In contrast to PFGE, RAPD and BT detect DNA elements with a relatively high evolutionary clock-speed. Obviously, this may be due to random accumulation of neutral mutations or to adaptation of the predominant strain to environmental changes. An epidemic MRSA strain that spread in diverse Spanish and Portuguese hospitals was found to display more pronounced genetic diversity, as estimated with PFGE, RAPD and BT. This could be evidential for an elevated number of replication rounds allowing more pronounced genetic adaptation. It is obvious that the typing data obtained with all whole genome typing methods largely agree with each other. The similarity of the predominant PFGE type 1 of the Iberian strains, which is clonally related to the type of the local outbreak strain, has also been confirmed with RAPD analysis (types MNA, MNJ) and BT (genotypes VI, XVIII) (fig. 1a,b). Short-term epidemicity does not seem to affect specific loci such as *mec* regulatory genes.

Long-term dissemination. All genotyping methods showed only limited genetic heterogeneity among the ancient MRSA strains originated from Europe (data not shown), a feature that has been described previously (2). The geographically and

temporally unrelated strains display a large number of polymorphisms upon *mec*A and Tn*554* probing. This genetic diversity is confirmed by the other whole genome typing techniques. Two distinct *mec* regulator types were identified (see table 2). The ancient MRSA strains, which lack the *mec*I and part of the *mec*R1 gene, are constitutive producers of PBP-2' (8). Our study supports this fact. Contemporary MRSA strains (isolated from the 1980s onwards) carry a complete set of *mec* regulator genes, also described previously (7). However, the recently isolated MRSA strains from New York (world wide collection), the New York local outbreak strains and their genetically related Portuguese strains lack the *mec*I and the 3'-part of the *mec*R1 gene. The *cna* gene also has limited value as an epidemiological marker for MRSA strains. In our study, this gene appeared to be randomly present or frequently lost.

Concluding remarks. During an outbreak, strains undergo short-term

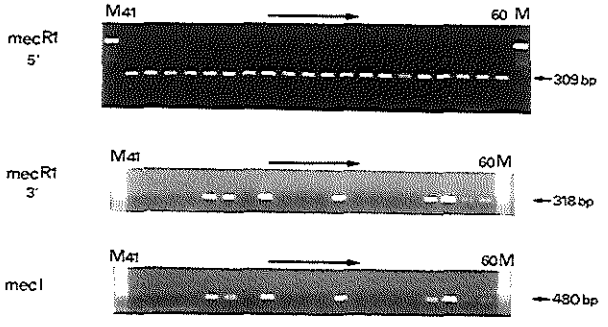


Figure 3. Distribution of *mec*-regulator genes in MRSA strains. Results of amplified regions are obtained from PCR with primer-pairs for the 5'- and 3'-end of the *mec*R1 gene and the *mec*I. Isolate numbers are indicated above the lanes. The outer lanes M contain molecular length markers. The amplicon-fragment length is indicated on the right.

dissemination, and the accumulated but nonetheless limited genetic variation can be measured effectively by RAPD analysis and BT. Nationwide dissemination of MRSA (1992-1994) occurred over a longer period. For this reason, an increasing number of polymorphisms could be detected, even with procedures such as PFGE. Comparative analysis of PFGE patterns obtained for isolates from a nationally dispersed clone of MRSA should be performed with caution: it might well be that the current guidelines for the interpretation of banding patterns are inadequate in this respect (24) (see also fig. 2b). Whole genome typing procedures detect increasing genetic variation as a result of pronounced dissemination of a MRSA clone. Short-term dissemination does not seem to affect specific loci such as *mec*A and *mec* regulator genes. The worldwide collection of MRSA strains displays polyclonality as a consequence of long-term dissemination and horizontal gene transfer (15), which confirms our current conclusion. Moreover, due to the continuous spread, distinct entities such as *mec*A and Tn*554* displayed the highest genetic heterogeneity, detected during our study.

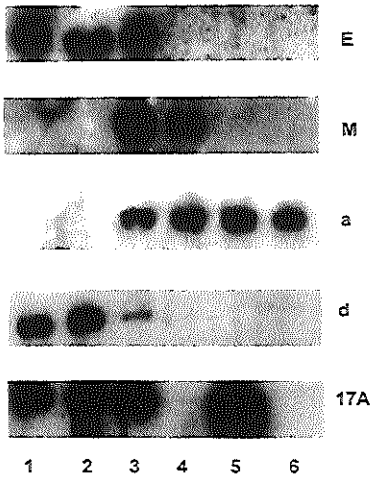


Figure 4. Representative example of autoradiograms obtained after hybridization with the strain-specific probes.
The combined results lead to a binary type. For instance, the hybridization pattern of strain 1 with the probes E, M, a, d, 17A reveal BT type V (10011); strain 2, BT type V (10011); strain 3, BT type VIII (11111); strain 4, BT type XX (01100); strain 5, BT type XIV (00101) and strain 6, BT type IX (00100).

Definitive genotyping for MRSA is not yet available. Our current study shows that, for specific purposes, such as epidemiological surveillance or outbreak investigation, the choice of molecular typing procedures should take into account the level of geographic dissemination.

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Dissemination of a single clone of methicillin-resistant *Staphylococcus aureus* among Turkish hospitals

Chapter

4

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SUMMARY

A collection of 39 methicillin resistant *Staphylococcus aureus* (MRSA) strains derived from six different hospitals in Ankara and one hospital in Bursa, Turkey, were analyzed by multiple genotypic. In agreement with the other genotyping assays, pulsed-field gel electrophoresis of DNA macro-restriction fragments identified genetic homogeneity among all MRSA isolates studied. It is concluded that a major clone of MRSA has spread through a large part of Turkey, causing longitudinally persistent colonization in all of the institutions surveyed.

INTRODUCTION

S. aureus has remained a major cause of nosocomial morbidity and mortality and methicillin-resistant *S. aureus* (MRSA) emerged at an alarming rate in the 1980s as an additional clinical problem (12, 15). Consequently, analysis of the dissemination of MRSA has been a research focus for the past decade. Resistance to methicillin, which is often accompanied by other forms of antimicrobial resistance, is usually a direct consequence of the acquisition of another penicillin binding protein (PBP-2a) gene, called *mec*A (10). In addition to *mec*A-regulatory genes like *mecI* and *mecR*, numerous auxiliary genes contributing to the overall level of methicillin resistance of a strain have been identified (7). Apparently the genetic background in which the *mec*A gene is embedded is of immediate importance to the clinical threat a given strain poses. Analysis of the genetic background by molecular typing techniques is important in the sense that specific DNA characteristics may be correlated with important phenomena such as strain epidemicity and virulence. International epidemiological surveillance requires reliable techniques, capable of differentiating independent strains from clonally related strains and molecular pheno- and genotyping techniques have been optimized for the purpose of studying the spread of MRSA (14, 30, 32). Regretfully, all procedures applied thus far, have specific experimental drawbacks. Multilocus enzyme electrophoresis (MLEE) ensures a high degree of typeability but is thought to be technically very demanding and to have limited discriminatory power (24). Pulsed-field gel electrophoresis (PFGE) has proven to be highly discriminatory for MRSA and it is suggested to be superior to other genotyping techniques. However, this method is fairly laborious and the DNA restriction patterns may be difficult to interpret (25). Moreover, interlaboratory standardization of PFGE is still problematic (3). Randomly amplified polymorphic DNA (RAPD) analysis has proven to be a rapid technique that yields epidemiologically valid results. But again, its interlaboratory reproducibility needs improvement (29). Thus, the continued need for accurate (geno)typing systems that can be applied in clinical laboratories is evident. It is suggested that only the combined application of various typing schemes allows accurate analysis of clonal relatedness among MRSA isolates (17, 28).

In the present study a collection of MRSA strains from six different hospitals in the Ankara region and a single hospital in the city of Bursa, both in Turkey, were analyzed with multiple genotyping procedures. The use of ribotyping, random amplification of polymorphic DNA, isolate specific DNA probes and PFGE enables assessment of the putative clonal spread of MRSA in this part of the world.

MATERIALS AND METHODS

Bacterial strains, cultivation and antibiotic susceptibility testing. A collection of 39 MRSA strains was obtained from seven different hospitals, of which six are located in Ankara (hospital B through G, for a more precise identification, see the footnote to Table 1) and one (hospital A) is within the city of Bursa. Ankara and Bursa are 380 kilometers apart. Colonies were selected on the basis of morphological characteristics, checked by Gram staining and tested for catalase and coagulase production. Coagulase production was assayed by the citrate-plasma tube technique (16). Swabs were inoculated directly onto 5% blood agar, mannitol salt agar and DNase test medium. Subsequently, the swabs were incubated in broth enrichment medium with 6.5% NaCl (37°C for 18 hours) or Stuart's transport medium (seven days at 4°C) (16). All solid media were incubated at 37°C for 16 hours, after which plates were placed at room temperature. Semi quantitative detection of susceptibility towards antibiotics, oxacillin and methicillin most specifically, was performed according to well-established disc diffusion procedures (1). All antibiotics were purchased from Oxoid (Unipath, Haarlem, the Netherlands), except for cephaperazone-sulbactam which was bought from BBL (Becton Dickinson, Etten-Leur, the Netherlands).

DNA isolation. For DNA isolation, bacteria were grown in suspension in Brain Heart Infusion (BHI) broth for 18 hours at 37°C. A volume of approximately 100 µl of a bacterial pellet was suspended in 150 µl of 25 mM Tris.HCl pH 8.0, 50 mM glucose, 10 mM EDTA. Lysostaphin (75 µl of a 100 µg/ml solution) was added and the mixture was incubated at 37°C for 1 hour. Spheroplasts were lysed by the addition of 1 ml 4 M guanidinium isothiocyanate, 50 mM Tris.HCl pH 6.4, 3 mM EDTA, 1% (W/W) Triton X100 (2). To immobilize and purify the DNA, 50 µl of a Celite suspension (0.2 µg/ml, Janssen Pharmaceuticals, Beerse, Belgium) was added. The entire mixture was shaken for 15 seconds and incubated at room temperature for 10 min. The pellet was washed once with 1 ml lysis buffer, twice with lysis buffer without EDTA and Triton X100, twice with 70% ethanol in water and finally once with acetone. Between 100 and 400 µl of 10 mM Tris.HCl pH 8.0, 1 mM EDTA was added and DNA was eluted by incubation at 56°C for 10 min. The supernatant containing the DNA was separated from the Celite by centrifugation. Stock solutions of bacterial DNA were adjusted to a concentration of 5 ng per 10 µl and stored at -20°C.

Table 1. Phenotypic and genetic data for the MRSA collection from Turkye.

Strain code	Anti-biogram	Hosp.	Ribo type	ERIC2 RAPD	PFGE	isolate-specific DNA probes d	17A	E	a	i	2B
A1	RRSIRRIR	A	A	A	Ia	+	-	-	+	+	+
A2	RRSRRRIM	A	A	A	Ib	+	-	-	+	+	+
A3	RRSRRRIM	A	A	A	Ic	+	-	-	+	+	+
A4	RRSRRRSR	A	A	A	Id	+	-	-	+	+	+
A5	RRSRRRRR	A	A	A	Ia	+	-	-	+	+	+
A6	RRSRRRIR	A	A	A	Ie	+	-	-	+	+	+
A7	RRSRIRIM	A	A	A	Ic	+	-	-	+	+	+
A8	RRSRRRSS	A	A	A	If	+	-	-	+	+	+
A9	RRSRRRIR	A	A	A	Id	+	-	-	+	+	+
A10	RRSRRRSS	A	A	A	Ic	+	-	-	+	+	+
A11	RRSIRRIR	A	B	A	Ig	+	-	-	+	+	+
A12	RRSRRRIM	A	A	A	Ih	+	-	-	+	+	+
B1	RRSRRRSS	B	A	A	Ii	+	-	-	+	+	+
B2	RRSRRRSM	B	B	B	Ij	+	-	-	+	+	+
B3	RRSRRRIM	B	A	A	Ik	+	-	-	+	+	+
B4	RRSIRRIR	B	A	A	Il	+	-	-	+	+	+
B5	RRSRRRSM	B	A	A	Il	+	-	-	+	+	+
B6	RRSRRSRR	B	A	A	Im	+	-	-	+	+	+
B7	RRSRRRSR	B	A	A	Ii	+	-	-	+	+	+
B8	RRSRRRRR	B	A	A	Im	+	-	-	+	+	+
B9	RRIRRRRR	B	B	A	In	+	-	-	+	+	+
B10	RRSRRRRM	B	A	A	Ii	+	-	-	+	+	+
B11	RRSRRRRR	B	A	A	Im	+	-	-	+	+	+
C1	SRIRRSRM	C	A	A	Io	+	-	-	+	+	+
C2	RRRRRRRR	C	A	B	Id	+	-	-	+	+	+
C3	SSRRRRRR	C	B	B	Ip	+	-	-	+	+	+
C4	RRRRRRRR	C	B	B	Iq	+	-	-	+	+	+
C5	SRRRRRRR	C	B	C	II	-	-	-	-	-	-
D1	RRSRRRRR	D	A	B	Io	+	-	-	+	+	+
D2	RRIRRRIM	D	A	A	Ir	+	-	-	+	+	+
D4	RRIRRRIR	D	A	A	Is	+	-	-	+	+	+
E1	SSRRRRSR	E	A	A	It	+	-	-	+	+	+
E2	RRRRRRRR	E	B	B	Iu	+	-	-	+	+	+
E3	RRRRRRRR	E	A	A	Iv	+	-	-	+	+	+
F1	RRSRRRIM	F	A	A	Iw	+	-	-	+	+	+
F2	RRRRRRIM	F	B	B	Ix	+	-	-	+	+	+
F3	RRSRRRIR	F	A	A	In	+	-	-	+	+	+
G1	RRSRRRIS	G	A	A	It	+	-	-	+	+	+
G2	RRSRRRIS	G	A	A	Iv	+	-	-	+	+	+

Note: Ribotypes differ by a single DNA fragment only. Affixed letters in the PFGE codes indicate (non)identity of banding patterns (see also Figure 4). All of the strains except C4 produced ß-lactamase. The antibiotics used for determining the antibiogram are from left to right: ciprofloxacin, ofloxacin, netilmycin, erythromycin, tetracyclin, imipenem, chloramphenicol and cotrimoxazol. Strains are denominated resistant (R), sensitive (S), moderately sensitive (M) and intermediately resistant (I). The originating hospitals are: A. Uledag University, Medical School, Dept. Infectious Diseases and Clinical Microbiology, Bursa; B. Ankara University, Medical School, Dept. Bacteriology, Ankara; C. Hacittepe, Medical School, Dept. Bacteriology, Ankara; D. Gazi University, Medical School, Dept. Bacteriology, Ankara; E. Turkish Ministry of Health, Yuksek Intisas Hospital, Ankara; F. Gulhane Military Medical Academy, Dept. Infectious Diseases and Clinical Microbiology, Ankara; G. Ankara University, Medical School, Cebeci Hospital, Ankara.

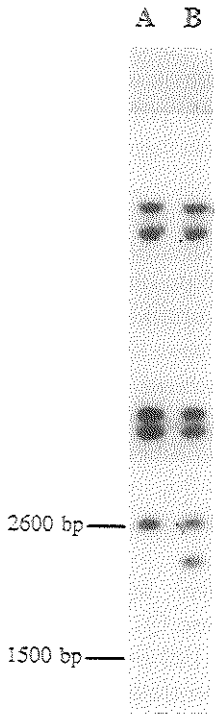
Ribotyping. Conventional ribotyping was performed by methods described previously (9). DNA (approximately 5 µg) was digested with the restriction enzyme *Eco*RI (Boehringer Manheim, Germany) according to the manufacturer's instructions. Restriction fragments were subsequently separated by electrophoresis in 0.8% agarose gels, which were Southern blotted onto Hybond N^+ membranes (Amersham, UK) (19). The *E. coli* 16S rRNA was amplified by the PCR and the amplicon was used as a probe for the detection of ribosomal restriction fragment length polymorphism. Hybridization was visualized using the chemiluminescent Amersham ECL kit (Amersham, UK).

Arbitrarily primed PCR. PCR was performed in a buffer system containing 10 mM Tris.HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100, 0.2 mM of the respective deoxynucleotide triphosphates, 50 pmoles of primer and 0.2 units Taq polymerase (Supertaq, HT Biotechnology, Cambridge UK), to which DNA is added (5 ng per amplification). The PCR mixtures were overlaid with 100 µl of mineral oil. Cycling was performed in Biomed PCR machines (Model 60) and consisted of the following steps: predenaturation at 94°C for 4 min and 35 cycles of 1 min 94°C, 1 min 25°C, 2 min 74°C. The primer used to discriminate *S. aureus* strains was ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'), which was previously shown to effectively discriminate among both methicillin-susceptible *S. aureus* and MRSA (11, 31, 35).

Agarose gel electrophoresis. Amplification products were separated by electrophoresis in 1% agarose gels (5 mm thick; Hispanagar, Sphaero Q, Leiden, the Netherlands). Gels were run in 0.5xTBE at a constant current of 100 mA for three hours. Prior to electrophoresis, samples were mixed with a 5x concentrated layer mix consisting of 50% glycerol in water and 0.8 mg bromophenolblue per ml. Thirty-five µl of the amplified material was loaded on the gel and a molecular weight marker was run in parallel with the RAPD samples. Gels were stained before electrophoresis by addition of 10 µl ethidiumbromide (10 mg/ml) to a total volume of 300 ml 0.5xTBE. Gels were photographed with a Polaroid MP4 Landcamera and Polaroid 57 High Speed films, exposure time was 0.125-0.25 seconds (diaphragm F5.6). Banding patterns visualized in this manner were indexed by capital lettering. Also single band differences led to the definition of a novel type.

Pulsed-field gel electrophoresis. PFGE was performed as described previously using a Chef Mapper (BioRad, Veenendaal, The Netherlands) in the auto-algorithm mode (3, 11). *S. aureus* was embedded in agarose blocks and treated with lysostaphin and proteinase K. Before electrophoresis the DNA was digested with the restriction enzyme *Sma*I (New England Biolabs, UK) and after PFGE banding patterns were visualized by ethidium bromide staining and photographed. Several strains from geographically diverse regions were included as control strains for the determination of resemblance of the Turkish strains to other important clonal types of MRSA. Interpretation was done according to previously determined standards (25). In practice, all bands were scored manually for presence or absence in a given PFGE fingerprint. As such the banding patterns were translated into a binary code,

consisting of pluses and minuses which is easily accessible for digital analysis. Phylogenetic trees were constructed with the help of computer programs accessible through the Internet. The PHYLIP program (Phylogeny Interference Package, University of Seattle, Washington, USA) version 3.5c was used to construct a phylogenetic tree. The maximum likelihood method was used to estimate phylogenetic distances between strains.



Hybridization studies with isolate specific DNA probes. Differentiation of staphylococcal strains can be based upon their reactivity towards DNA probes capable of discriminating different staphylococcal genome compositions. These probes have been developed and described recently (33) and can be used for epidemiological tracking of MRSA. Southern blots of purified staphylococcal DNA were prepared as described above (ribotyping section). Probe labeling, hybridization and detection was performed with the ECL® direct labeling and detection systems (Amersham Life Science, Buckinghamshire, England), according to the manufacturers protocols and according to a previous publication (33). Six different DNA probes were applied, hybridization was scored with a plus or a minus according to the presence or absence of hybridization signal, respectively.

Figure 1 Ribotyping of MRSA from the Ankara region and Bursa. The picture shows the two types as encountered in the seven institutions, in the left lane pattern A is observed, the right lane shows pattern B. On the left, the sizes of two molecular markers are indicated.

RESULTS

All data obtained for the strains gathered in the Ankara and Bursa regions are summarized in Table 1. Besides the strain codes and the originating institution, data concerning ribotyping, arbitrarily primed PCR and the isolate specific DNA probing are given. A presumptive indication of the PFGE classification is also provided (see below for further detail). Upon antibiotic susceptibility testing it became apparent that all strains produced ß-lactamase (except for strain C4) and were uniformly resistant to vancomycin, tobramycin, penicillin, cefuroxim, cefataxim, ceftazidime, piperacillin, flucloxacillin, cefipime, metronidazol, aztreonam and cephaperazone-sulbactam. Towards the other antibiotics tested (see Table 1) differential susceptibility was determined although towards erythromycin and tetracyclin only resistant and intermediately resistant phenotypes were determined. There appeared to be no close

relationship between antibiogram and the originating hospital.

Only two different ribotypes could be detected (see Figure 1). Either five (type A) or six (type B) DNA fragments hybridized to the rRNA gene probe. Apparently, both types are quite closely related. The B type was documented in 8 out of 39 strains (prevalence of 21%) and occurred in 5 out of 7 institutions. In hospital III the incidence of type B was 60%, possibly indicating endemic spread among the patients nursed here. In hospitals G and D the B type was not encountered, which may be due to the small number of strains derived from these institutions (see Table 1).

Arbitrarily primed PCR using primer ERIC2 revealed three different patterns only (Figure 2). The two most frequently observed patterns (A and B) differed by a single band, whereas the most aberrant (C) type occurred only once (strain C5, hospital III). Although there appeared to be no 100% overlap, the AP PCR B type seemed to be linked to a B ribotype in the majority of cases, demonstrating the existence of two closely related lineages of the same clone (see also discussion). In accordance with this latter observation, in hospital III the AP PCR B type again reached an incidence of 60%.

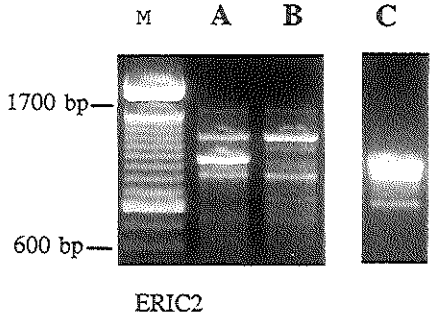


Figure 2. Arbitrarily primed PCR using the ERIC2 primer of MRSA from the Ankara region and Bursa. The picture shows patterns A through C, highlighting the fact that A and B are highly similar (single band differences). The lane marked M contains molecular length markers (100 basepair ladder, Gibco BRL, Breda, the Netherlands), the size of some of which is given on the left.

Probing genomic DNA of the MRSA strains with DNA probes capable of differentiating among strains of this bacterial species (33) corroborated the AP PCR and ribotyping data. All strains behaved in an identical manner: the digital + / - code based on the hybridization studies was identical for all of the strains except for the already mentioned strain C5 from hospital III, which did not react with any of the probes applied for typing.

Pulsed field gel electrophoresis was carried out to obtain final proof on the observed homogeneity of this cluster of MRSA. The actual gel pictures are shown in Figure 3.

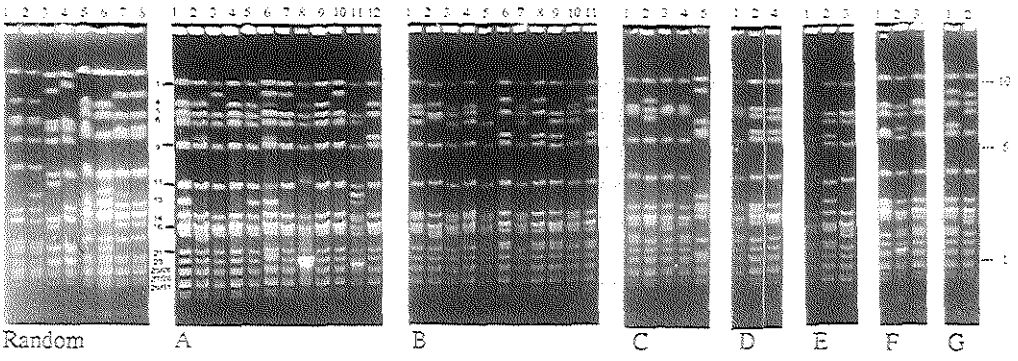


Figure 3. Pulsed field gel electrophoresis of *Sma*I macrorestriction fragments of MRSA from the Ankara region and Bursa. The numbering above the lanes and the lettering under the separate panels indicate the nature of the strains (see also Table 1). Figures between the outmost leftward panels indicates DNA fragment-numbering as in Table 2. The panel on the left (Random) shows the analysis of some reference strains. The first two from the left represent isolates from the Iberian clone (see ref. 20 as well), whereas the other isolates represent the major Polish clones AA (lanes 3 and 4) and BB (lanes 5-8) (see also ref. 26). Note that some banding features of the Polish BB type are shared with the Turkish strains, identity is not obvious however. On the right three molecular length markers are given (10, 5 and 1 megabases, from top to bottom of the figure).

Overall, 31 different, position-based bands could be observed which were scored for absence or presence in each of the strains (data not shown). Numbers of some of the bands are indicated in Figure 3. Based on this band presence score a phylogenetic tree could be constructed (Figure 4). Except for strain C5 all of the MRSA isolates fell within a 10% difference margin compared to the average homology value of 90%, indicating that in the order of three bands difference was the maximal divergence (3 out of 31 bands equals approximately 10%). It is interesting to note that all of the strains from the Bursa hospital cluster at the top of Figure 4 (see boxed region). This indicates that resident strains, being established in a given hospital, evolve, albeit slowly, and may in the end present as a separate, novel genetic entity. Figure 3 shows that all of the "control strains" included in the present study and being representatives of some of the major European MRSA clones, are clearly more distant at the PFGE type level. Additional studies into the precise nature of the *mec* region may shed light on the archaeal type from which the present Turkish MRSA descended (10).

Consequently, it can be concluded that 38 out of 39 of the MRSA isolates belong to a single, genetically homogeneous cluster of strains. Only strain C5 presents aberrantly: this isolate possibly reflects and imported case of MRSA disease and not local nosocomial acquisition of the resident strain. Strains from the single hospital that is remote from the others, tend to cluster separately, but still the genetic distance to the Ankara strains is limited.

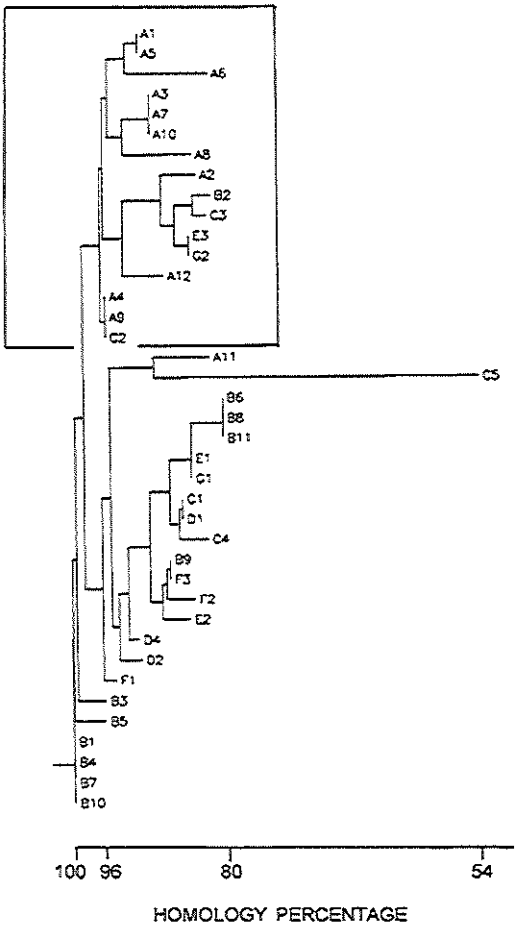


Figure 4. Phylogenetic tree displaying the grouping of the MRSA strains from the Ankara region and Bursa based on banding pattern score after PFGE analysis. Note that strains from hospital I (A1 through A12) group on top of the figure (boxed region).

DISCUSSION

Primarily clonal spread of methicillin-resistant *S. aureus* has been suggested (11), whereas other reports suggest that multiple episodes of horizontal gene transfer among staphylococci have occurred and multiple major MRSA clones exist (15). Examples of the latter phenomenon where major MRSA clones successfully spread over large geographic areas have recently been documented. Nationwide dispersal of a given MRSA genotype has been demonstrated for several West European countries such as Belgium (21, 22), Poland (26) and Germany (37). In Portugal and Spain a major MRSA type crossed borders and became highly prevalent in both countries (20). The United Kingdom also has a history of major episodes of colonization and infection caused by epidemic MRSA (5, 6). Outside of Europe other epidemic MRSAs have been described as well (18, 23). On the contrary, several institutions describe the increase in number of different MRSA genotypes as encountered among their patients (4, 13, 27). This may be explained by an increase in the number of people in the general population that is colonized with MRSA. This conclusion was underscored by a recent pan-European surveillance on MRSA prevalence where it was emphasized that both epidemic and non-epidemic MRSA exist and that these types may co-exist in the same environment (36). The factors that determine whether a strain

becomes epidemic are largely unknown (8, 34) but it is obvious that large differences in MRSA "behavior" can be observed between different clinical settings. For that reason it is worthwhile to continue the search for epidemic MRSA and, after identification, study those particular strains in more detail with respect to "spreading determinants". We here show that in hospitals in the Ankara and Bursa regions a major MRSA clone circulates. With the help of genetic typing procedures, each of which has reliably and reproducibly been shown to enable the detection of genetic polymorphisms among MRSA, genetic homogeneity among the Turkish MRSA is well established.

Apparently, the MRSA involved is very well adapted to the clinical settings and capable of dissemination and local maintenance. It remains to be seen whether the "Turkish clone" is genetically similar to one of the other major European MRSA types, although the preliminary data reveal that no obvious homologies at the DNA level seem to exist with two Polish and the Iberian clones of MRSA. Preliminary data on the genetic resemblance of strains from Istanbul (Turkey) which is 440 km away from Ankara already indicate that also in the Turkish capitol strains belonging to the same clonal lineage can be identified (C. Acuner and A. van Belkum, unpublished data). When analyzing the strains from Bursa and comparing them to the ones from Ankara subtle genetic differences are observed: strains from Bursa tend to cluster separately, but not distantly. Apparently, some sort of local adaptation or even small scale evolution occurs once strains become geographically isolated.

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Binary typing of *Staphylococcus aureus* in veterinary microbiology

Chapter

5

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SUMMARY

Thirty-eight bovine mammary *Staphylococcus aureus* isolates from diverse clinical, temporal, and geographical origins were genotyped by pulsed-field gel electrophoresis (PFGE) after *Sma*I digestion of prokaryotic DNA and with binary typing. Seven pulsed-field types and four subtypes were identified, as were 16 binary types. Genetic relatedness of the strains was documented concordantly by both techniques. Based on practical and epidemiological considerations, binary typing was preferred. Genotypes of bovine isolates strains were compared to 55 previously characterized genetically unique human *S. aureus* strains through cluster analysis of binary types. The distinct genetic clusters were predominantly associated with a single host species, but some lineages contained strains of both human and bovine origin. We conclude that PFGE and binary typing can be successfully applied for genetic analysis of *S. aureus* isolates from bovine mammary secretions, for the elucidation of population structure of bacteria within and between host species, and for identification of sources and transmission routes of bovine *S. aureus*.

INTRODUCTION

Staphylococcal infections are of major importance to veterinary and human medicine. *Staphylococcus aureus* is one of the most significant pathogens causing intramammary infections in dairy cattle worldwide (6, 13, 28). In human medicine, *S. aureus* has remained a major cause of community-acquired as well as of nosocomial infections and in the last four decades, the increasing prevalence of methicillin-resistant *S. aureus* (MRSA) strains has become an additional problem (4, 7). Staphylococcal strains may vary considerably in virulence and epidemiological potential. To control the spread of staphylococcal infections, sources of contamination and mechanisms of transmission must be identified. Detailed pathogenic and epidemiological studies depend on the availability of typing systems that differentiate between strains belonging to the same bacterial species.

In veterinary microbiology, both phenotypic techniques (phage typing (2), biotyping (10), multilocus enzyme electrophoresis (15) and genotypic approaches have been applied for characterization of bovine *S. aureus* strains. Genotypic methods include gene typing systems, such as detection of coagulase gene polymorphism (30) and ribotyping (2), and genome typing techniques, such as arbitrarily primed PCR (18) and pulsed-field gel electrophoresis (19). So far, binary typing has not been applied to *S. aureus* strains of bovine origin. Binary typing was used for the characterization of *S. aureus* strains in human medicine (22, 33, 35, 36) and has proven to be a reproducible and stable library typing method with excellent discriminatory abilities. It has the additional advantage of producing a simple binary output (36). The purpose of this study was to determine whether

binary typing is a suitable technique for the differentiation of *S. aureus* strains originating from bovine mammary secretions. In addition, a collection of bovine isolates was compared to a collection of human isolates, including methicillin-resistant strains, to explore the population structure of strains from cattle and humans as determined by binary typing.

MATERIALS AND METHODS

Bacterial isolates. This study included 38 bovine *S. aureus* isolates collected from eight dairy herds in The Netherlands between May 1997 and February 1999. Three herds (I, II and III) were involved in a longitudinal survey on population dynamics of intramammary infections. In those herds, milk samples were routinely collected from all udder quarters of each cow at intervals of 3 weeks for a period of 81 weeks. Samples from the other five herds were submitted to the diagnostic laboratory of the Animal Health Service (Deventer, The Netherlands) as part of a dairy health improvement scheme. Bacteria were cultured from milk samples according to National Mastitis Council standards (14) and identified at the species level as described previously (17). Isolates were stored frozen until further use. Isolates were selected to represent different geographical and temporal origins (Table 1).

Binary typing data on 55 human *S. aureus* isolates from diverse geographic and temporal origins in the United States and The Netherlands were used (Table 2). Human collections include MRSA strains (n=37) and methicillin-susceptible *S. aureus* (MSSA) strains (n=18) and have been described in detail before (31, 36).

PFGE. Restriction with *Sma*I (Roche, Mannheim, Germany) of genomic staphylococcal DNA and subsequent separation of the DNA macrorestriction fragments was performed by contour-clamped homogeneous electric field (CHEF) PFGE as described before (29). Macrorestriction patterns were analyzed both visually and by computer-aided methods. Visual interpretation of banding patterns was done following the type definition guidelines suggested by Tenover et al. (32). Strains with identical restriction profiles were assigned the same type and indicated with a capital letter. Strains that differed from main types by one to three band shifts, consistent with a limited number of genetic events, were assigned subtypes, and indicated with a numeral suffix. Strains with more than three such differences were considered to be different types. Banding patterns were digitized with a Hewlett-Packard Scanjet IIcx/T scanner and stored as TIFF files. Patterns were analyzed using GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium) to calculate Dice coefficients of correlation and to generate a dendrogram by unweighted pair group method using arithmetic averages (UPGMA) clustering.

Table 1. Summary of epidemiological data, PFGE typing data, and binary typing results for 38 bovine *S. aureus* strains

Herd	Isolate no.	Cow no. quarter no.[a]	Collection period	PFGE type	Binary code[b]
I	1	75-1	August 1997	A	001111111111101
	2	75-2	August 1997	B	001111111111101
	3	75-4	August 1997	B	001111111111101
	5	78-3	August 1997	B.1	001111111111101
	6	63-3	June 1997	B	001111111111111
	7	77-4	October 1997	C	000010011000001
	8	67-3	January 1998	D	101010011110011
	9	74-4	December 1997	D	101010011110011
	10	75-4[c]	December 1997	D	101010011110001
	12	25-4	May 1997	D	101010011110011
	13	25-4	May 1998	D	101010011110001
	14	18-4	November 1998	B.2	001111111110001
	15	53-2	November 1998	B	001111111110001
	16	90-3	November 1998	B	001111111110011
II	17	11-3	July 1997	E	000110011010011
	18	18-3	July 1997	E.1	010110011010011
	19	99-2	July 1997	E	000110011010001
	20	108-3	July 1997	F	0010110111111111
	21	47-3	May 1998	D	101010011010001
	22	29-4	August 1997	D	101010011010011
	23	70-3	April 1998	D	101010011010011
	24	21-4	May 1998	E	000110011010011
III	25	25-3	July 1997	C	000010011010011
	26	95-1	July 1997	E	000110011010011
	27	46`-4	December 1997	C	000010011010011
	28	29-1	May 1998	E	000110011010011
	29	29-1	July 1998	E	000110011010011
	30	31-3	May 1998	E	000110011010011
	31	31-3	July 1998	E	000110011010011
	32	86-2	May 1998	E	000110011010011
	33	86-2	July 1998	E	000110011010011
	34	NA	May 1998	E	000110011010011
	35	NA	July 1998	E	000110011010011
IV	36	Ada126-4	February 1999	E	000110011010011
V	37	9363-3	February 1999	E.2	000111111110011
VI	38	205-4	February 1999	E	000110011010011
VII	39	68-?	February 1999	D	101010011010011
VIII	40	Klara-4	February 1999	G	001011111111101

[a] Udder quarter numbers: 1, right front; 2, left front; 3, right rear; 4, left rear.
[b] Overall results after hybridization with 15 strain-specific DNA probes (AW-1 trough AW-15) developed for typing of human *S. aureus* strains (36)
[c] New animal assigned same cow number (75) as cow from which isolates 1, 2 and 3 were collected

Table 2. Characterization of human *S. aureus* collections from which binary types are used in this study.

Collection no.	Geographic origin	Isolate no.[a]	Description of collection	Reference(s)
1	United States	41-66	Community-acquired MRSA strains from a New York City hospital (n=26)	41
2	United States (CDC[b])	67-80	Selection of geographically diverse strains from multicenter collection of MRSA strains (n=5) and MSSA strains (n=9)	38, 42
3	The Netherlands	81-85	MSSA strains isolated from healthy persistent nasal carriers (n=5)	42
4	The Netherlands	86-95	MRSA strains (n=6) and MSSA strains (n=4) from outbreaks in Dutch hospitals and nursing homes	42

[a] Isolate numbers are those used in Fig. 4.
[b] CDC, Centers for Disease Control and Prevention

Binary typing. Macrorestriction fragment patterns obtained with PFGE were Southern blotted onto Hybond N$^+$ membranes (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) and hybridized with strain-differentiating DNA probes designed for binary typing of human *S. aureus* strains (36). Labeling, hybridization, and detection of the probes were performed with ECL direct labeling and detection systems, according to the manufacturer's protocols (Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom). Hybridization of 15 DNA probes was scored with a 1 or a 0 according to the presence or absence of a hybridization signal, resulting in a 15-digit binary code for each *S. aureus* isolate. A dendrogram was constructed using hierarchical cluster analysis (SPSS 8.0 for Windows, SPSS Inc.).

RESULTS

PFGE. Among 38 bovine isolates, seven pulsed-field types and four subtypes were identified through visual interpretation of gels (Fig. 1; Table 1). Three PFGE types (A, F, and G) and all subtypes (B.1, B.2, E.1, and E.2) were identified only once, while PFGE types C, D and E were found in two, three and four herds, respectively.

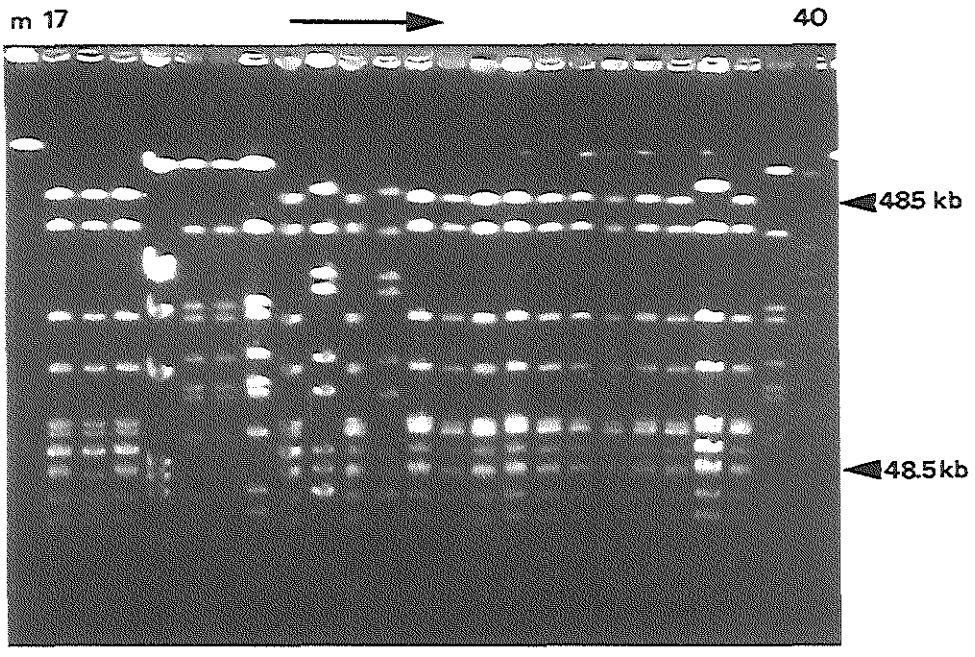

Figure 1. Example of PFGE of *Sma*I macrorestriction fragments of bovine *S. aureus* isolates, showing isolates 17 to 40. Molecular sizes are indicated on the right.

GelCompar analysis of PFGE results defined more clusters than visual interpretation. Depending on the level of genetic relatedness 13, 11 and 8 clusters were identified for 95, 90, and 80% similarity settings, respectively (Fig. 2). The visually identified PFGE type B was divided in four (95%) or two (80%) separate clusters, while PFGE type D was divided into three (95%), two (90%), and one (80%) cluster(s). In the GelCompar analysis, visual PFGE types E and E.1 were grouped together at 95% and E, E.1, and E.2 were grouped together at 90% genetic similarity.

Binary typing. All bovine isolates were typeable by the binary system (Table 1). Out of 15 probes designed for typing of human *S. aureus* strains, four hybridized to all bovine isolates (AW-5, AW-8, AW-9 and AW-15), while all other probes hybridized to at least one bovine isolate. Genetic relatedness of strains based on binary typing was depicted in a dendrogram (Fig. 3a). For 95, 90, and 85% similarity, respectively, eight, six and three clusters of strains were identified. Binding of probe AW-14 showed a low level of reproducibility among epidemiologically related bovine strains. Therefore, a separate dendrogram excluding AW-14 was constructed (Fig. 3b), reducing the number of clusters to six at 95% similarity.

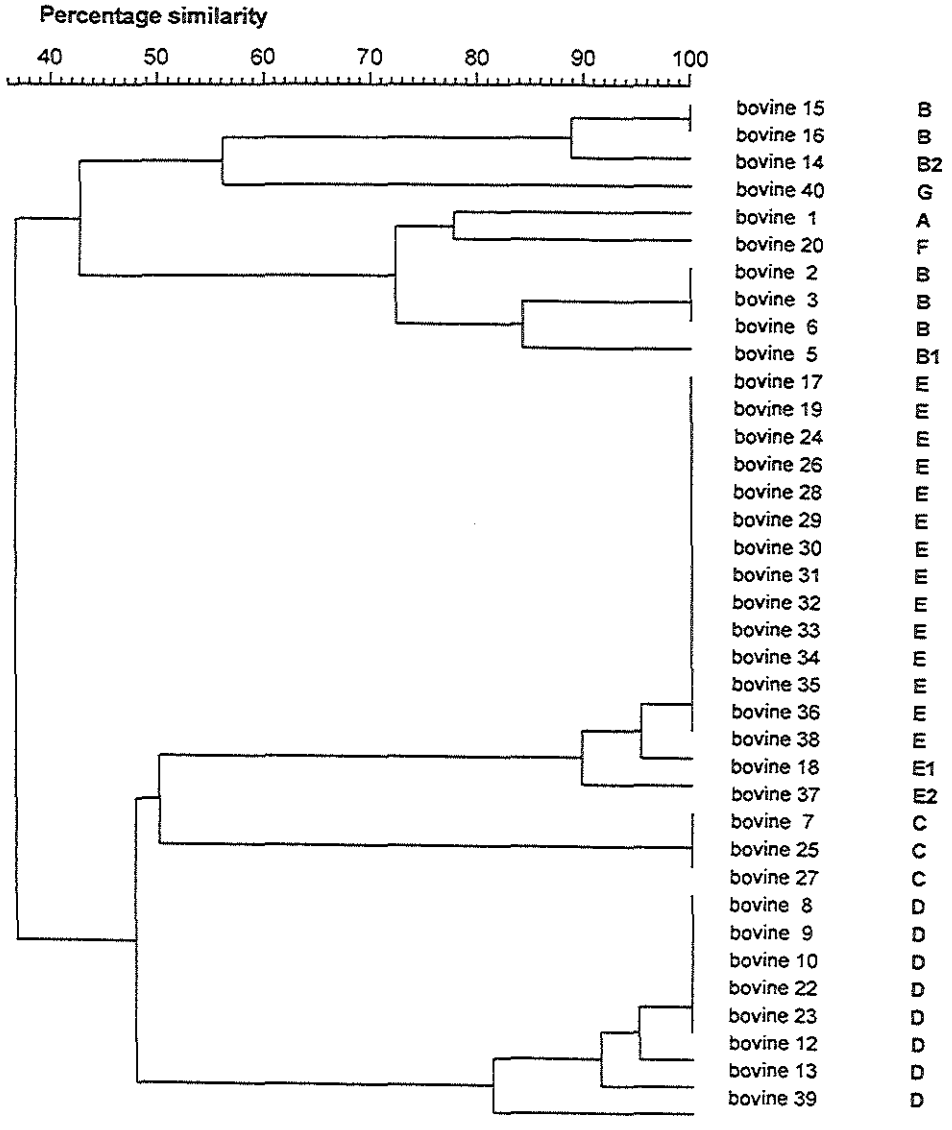

Figure 2. Dendrogram showing the level of similarity between *Sma*I macrorestriction patterns of 38 bovine *S. aureus* isolates (isolate numbers correspond with those indicated in Table 1) as determined by PFGE and subsequent GelCompar analysis of digitized photographs. Scale indicates level of genetic relatedness within this set of strains. Capital letters indicate PFGE types based on visual interpretation of PFGE results.

Concordance between PFGE and binary typing. PFGE types assigned to the isolates were compared with binary types. General agreement was found between both techniques, but with some discrepancies. Several visually identified separate PFGE types were grouped together by binary typing (e.g., A, three B strains, B.1, and G at 95% binary similarity; E, E.1 and two out of three C strains at 95% similarity; B, B.2, and E.2 at 90% similarity). Other PFGE types were divided into multiple binary clusters that differed by two or three probes (e.g., B into two binary clusters at 90% similarity) (Fig. 3a). Concordance of delineation of genotypically related clusters as determined by PFGE and binary typing improved when probe AW-14 was excluded (Fig. 3b).

Within herd heterogeneity. Genetic heterogeneity among *S. aureus* strains recovered from bovine mammary secretions was observed within and between herds. Strains from herd I (n=16) were divided into four PFGE types (A to D) and subclonal variation was observed in PFGE type B (subtypes B.1 and B.2). In herd II (n=8) three PFGE types (D to F) were identified, with subclonal variation in PFGE type E (subtype E.1). In herd III (n=11), two PFGE types (C and E) occurred. In strains obtained from five herds that were not related to each other or herds I, II, and III, three PFGE types and one subtype were identified (D, E, E.2, and G), demonstrating that both heterogeneity and homogeneity between herds exists. Heterogeneity based on binary typing parallels heterogeneity of PFGE types for all herds.

Comparison of bovine and human strains. Binary types of bovine isolates presented in this study were compared to a well-defined collection of human *S. aureus* strains that had been typed previously by the same method (36). Most strains clustered as host-specific clones, and full identity of the 15-digit binary code of bovine and human strains was never observed (Fig. 4). At 95% similarity, human isolate 61 clustered together with bovine isolate 6 (one-digit difference, probe AW-11), and human isolate 62 clustered with bovine strains 1 to 5 (one-digit difference at probe AW-1) and bovine isolate 40 (two-digit difference). At 90% similarity, these bovine and human strains formed one cluster that also included human isolate 53. Human strains within this cluster differed from bovine strains in the same cluster by three digits, with differences associated with 6 out of 15 probes. Human isolate 82 clustered together with all bovine type D at 90% similarity, as did human isolate 50 with bovine isolate 20. Human strains 50, 53, 61, and 62 were community acquired MRSA strains from a New York City hospital (Table 2). Human isolate 82 was a MSSA strain isolated from a persistent nasal carrier in The Netherlands. Bovine strains that clustered with human strains originated from four Dutch dairy herds that were epidemiologically unrelated to each other or the human sources of *S. aureus* included in the comparison.

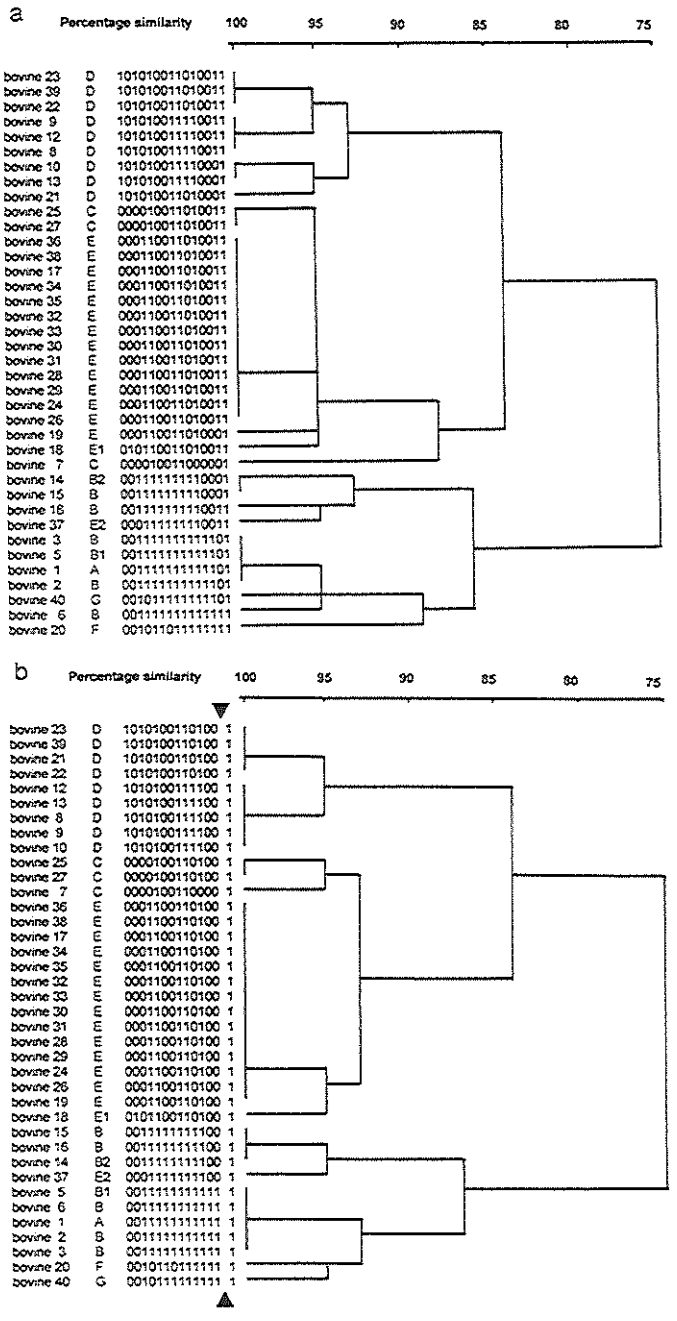

Figure 3. Dendrogram showing the grouping of 38 bovine *S. aureus* isolates on the basis of hybridization scores after binary typing with probes AW-1 to AW-15 (a) and after omission of probe AW-14, which is associated with hypervariable regions on the bovine staphylococcal genome (b). Isolate number, visual PFGE type, and binary code are given for all isolates. Scale indicates level of genetic relatedness within this set of isolates.

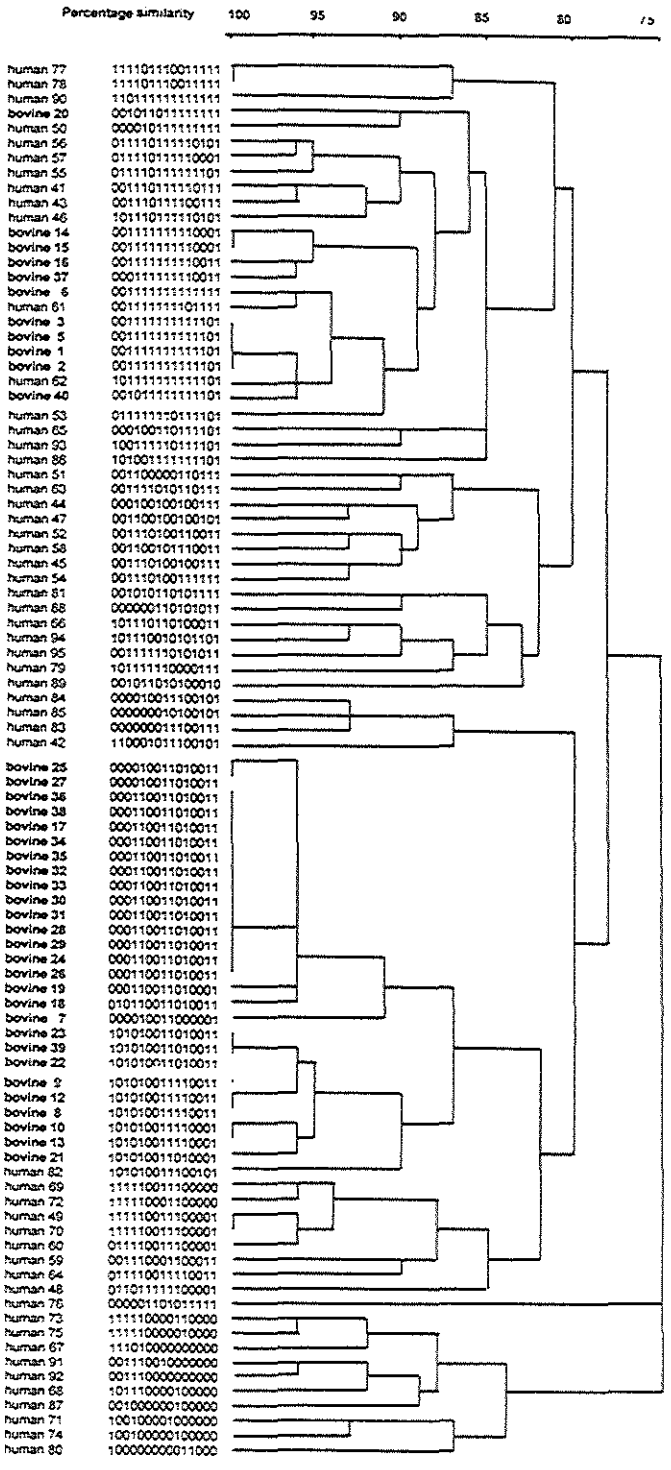

Figure 4. Dendrogram showing the grouping of 55 unrelated human *S. aureus* strains described previously (36) and 38 bovine *S. aureus* isolates on the basis of hybridization scores after binary typing with 15 DNA probes. Strain numbers and binary codes are shown for all strains. Scale indicates level of genetic relatedness within this collection of strains.

DISCUSSION

PFGE and binary typing. Whole genomes of pathogenic bacteria are currently being sequenced, and data obtained from comparison of these genomes suggest plasticity of the bacterial genome. PFGE is a well-known and powerful method for detection of such flexibility among *S. aureus* strains (5, 29). Binary typing is a recently developed potential library typing system for the characterization of *S. aureus* strains and produces a simple binary output (36). In this study, PFGE-profiles and binary codes for 38 isolates derived from bovine mammary secretions were determined and compared. Seven main PFGE types and four subtypes were identified visually. Computer-aided cluster analysis identified more distinct types, depending on the cutoff value. The relevant level of discrimination between clusters of strains depends on the bacterial epidemiology and population structure. The PFGE results must be analyzed in light of the epidemiological background (5, 29). One visually identified PFGE type, type B, was subdivided over multiple clusters after UPGMA analysis, even at low genetic similarity levels (Fig. 2). Strains were from similar clinical and geographical backgrounds, but subdivision may be related to different temporal origins of the samples (Table 1). Discrepancies between visual and computer-aided interpretation, especially occurring with the comparison of multiple PFGE gels (reproducibility) are a drawback of pulsed-field typing and limit its usefulness as a routine diagnostic technique for large numbers of bacterial isolates.

Binary typing was combined with PFGE, but binary typing can be performed as a single typing technique because of an excellent discrimination power (36). Binary typing yielded 16 binary codes clustered in three to eight clones, depending on levels of genomic similarity. The relevant level of discrimination and suitability of individual probes are subject of further study. Probes AW-12 and AW-13 yielded identical results, while probes AW-5, -8, -9, and -15 hybridized to all bovine strains and did not contribute to the discriminatory power of the typing system. A larger collection of bovine strains should be studied to determine the informative value of these probes for differentiation of bovine *S. aureus* strains.

Concordance between PFGE and binary typing. Several PFGE types were subdivided by binary typing. Binary codes within a PFGE type often differ by no more than one digit, frequently associated with probe AW-14 (Table 1; Fig. 3a). The observed discrimination within PFGE types may therefore be related to the detection of hypervariable domains on the genome of bovine *S. aureus* strains with probe AW-14. Similar hypervariability or inconsistent presence of probe-binding sequences has been described for epidemiologically and genetically related human *S. aureus* strains (34). The results could imply that probe AW-14, which is stable for typing of human *S. aureus* strains, is not stable for typing of *S. aureus* of bovine origin. On the other hand, probe AW-14 could be used to study short-term genome evolution in bacterial populations of bovine origin (35).

When binary code differences caused by probe AW-14 results are ignored, even closer agreement between binary typing and pulsed-field typing is obtained, but some one-digit differences within similar PFGE type strains remain (Fig. 3b). PFGE types C isolated from herds I and III differ by one digit, which was associated with probe AW-11. This genotypic difference can be related to different geographical origins. For PFGE type D, differences in geographical origin exist.
Some binary clones are subdivided by PFGE. Since strains within these binary types were of similar geographical and temporal origin, the stability of binary typing seems epidemiologically superior in these cases.

Within-herd and between-herd heterogeneity. PFGE and binary typing differentiated strains within and between herds. Similar results were obtained by various other studies (1, 11, 15, 18, 19, 21, 30). In all studies, including the present one, a limited number of predominant types was found both within herds, in agreement with the contagious nature of *S. aureus* mastitis (18), and between herds, suggesting that certain variants present in the environment may have a predilection for causing intramammary infections (1, 11, 30).
Subclonal heterogeneity within herds may be due to temporal evolution. Herds were selected for inclusion in the longitudinal survey based on a history of the presence of the pathogen in the herd for more than 1 year. The study period covered an additional 18 months, allowing for further genetic diversification (35). Similar subclonal genetic variation over time has been described for DNA macrorestriction patterns from human *S. aureus* strains (24).

Comparison of bovine and human strains. Out of 55 human strains and 38 bovine strains, 5 human and 16 bovine strains belonged to clusters sharing 90 to 95% similarity, as determined by binary typing. At higher similarity levels, all clones were host species specific. Similar results were obtained by Kapur et al. (15) and by Lopes et al. (20). The results are consistent with the concept of host specificity among *S. aureus* clones and imply that successful transfer of bacteria between humans and cattle is not a frequent event (15). However, several studies are available that suggest that transfer of bacteria between humans and cows is possible (12, 26). Those studies mostly focus on the role of humans as source of infection for dairy cattle. Another reason to be concerned about interspecies transfer of *S. aureus* is the routine use of antibiotics in dairy herd management (13, 27, 28). In farms with *S. aureus* mastitis problems, oxacillin is used as a dry cow therapy (8). Resistance to the closely related antibiotic methicillin is rare in bovine *S. aureus* (20), but has been reported in New York State (25), Europe (9), and Japan (cited in reference 16). Widespread use of oxacillin could promote the selection of resistance clones (7). If interspecies transfer occurs, methicillin resistance in bovine strains may occasionally contribute to increasing prevalence of MRSA strains in humans. Since binary typing is a library system that can be applied to *S. aureus* strains originating from humans and cattle, it is a useful tool in monitoring origins of MRSA strains and putative interspecies transfer of *S. aureus*. Addition of probes to test for the presence of the *mecA* gene in the bovine typing system would

furthermore allow monitoring of MRSA prevalence in veterinary diagnostic laboratories.

Conclusion and future developments. This study shows that both PFGE and binary typing can be successfully applied to characterize *S. aureus* strains of bovine mammary origin. Binary output was easier to interpret than banding patterns generated by PFGE, and binary typing seemed superior to PFGE in clustering strains from similar epidemiological background. As a library typing system, binary typing facilitates the comparison of *S. aureus* strains of bovine and human origins from worldwide collections, analysis of clonal relatedness and host specificity, and monitoring of interspecies transfer. In this study, genetically related clusters of strains of human, bovine and mixed origins occurred. We conclude that binary typing in particular is a technique that is suitable for use in veterinary microbiology.

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Validation of binary typing for *Staphylococcus aureus* strains

Chapter 6

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SUMMARY

Most of the DNA-based methods for genetic typing of *Staphylococcus aureus* strains generate complex banding patterns. Therefore, we have developed a binary typing procedure involving strain-differentiating DNA probes which were generated on the basis of randomly amplified polymorphic DNA (RAPD) analysis. We present and validate the usefulness of 15 DNA probes, according to generally accepted performance criteria for molecular typing systems. RAPD analysis with multiple primers was performed on 376 *S. aureus* strains of which 97% was methicillin-resistant (MRSA). Among the 1,128 RAPD patterns generated, 66 were selected which identified 124 unique DNA fragments. From these amplicons, only 12% turned out to be useful for isolate-specific binary typing. The nature of the RAPD-generated DNA fragments was investigated by partial DNA sequence analysis. Several homologies with known *S. aureus* sequences and with genes from other species were discovered; however, 87% of the probe sequences are of previously unknown origin. The locations of most of the DNA probes on the chromosome of *S. aureus* NCTC 8325 were determined by hybridization. Seven fragments were randomly dispersed along the genome, five were clustered within the 2500- to 2600- kb position of the genome, and the remaining four did not recognize complementary sequences in *S. aureus* NCTC 8325. A total of 103 *S. aureus* strains (69% MRSA) were used for the validation of the binary typing technique. The 15 DNA probes provided stable epidemiological markers, both in vitro (type consistency after serial passages on culture media) and in vivo (comparison of sequential isolates recovered from cases of persistent colonization). The discriminatory power of binary typing (D=0.998) exceeded that of pulsed-field gel electrophoresis (D=0.966) and RAPD analysis (D= 0.949). Reproducibility, measured by analyzing multiple strains belonging to a multitude of different epidemiological clusters, was comparable to that of other genotyping techniques used. Contribution of the DNA probes to the discriminatory power of the system was analyzed by comparison of dendrograms. This study demonstrates that binary typing is a robust tool for the genetic typing of *S. aureus* isolates.

INTRODUCTION

Staphylococcus aureus has remained a prime pathogen of nosocomial and community-acquired infections. Worldwide, the increasing prevalence of multiresistant *S. aureus* has become an additional problem (4, 20, 25). Consequently, the epidemiology of *S. aureus* infections needs to be studied, and for this purpose multiple typing techniques based on the detection of DNA polymorphisms have been developed and optimized (3, 22). Nucleotide sequence variations among *S. aureus* strains can be identified by a number of techniques, varying from pulsed-field gel

electrophoresis (PFGE) (39, 46) to randomly amplified polymorphic DNA (RAPD) analysis (37). However, these techniques generate complex banding patterns which lack generally accepted interpretation criteria (8, 36). Consequently, comparison of large numbers of fingerprints is very tedious and has little validity beyond the individual laboratory (8, 38, 42). Therefore, we have sought to develop less tedious typing systems that can be interpreted unequivocally. We have identified relatively unique domains within the staphylococcal genome on the basis of RAPD analysis that could be targets for such a typing system. Strain-specific DNA probes which produce a simple binary output were isolated by using hybridization assays. This collection of probes thus constitutes a so-called library typing system that can elucidate genetic polymorphism and clonal relatedness among *S. aureus* strains (45, 46).

In this study, the DNA probes were sequenced and homologies with known sequences and their locations on the physical map of *S. aureus* NCTC 8325 (29) were determined. The performance of this binary typing system was validated by using the evaluation criteria as proposed by Struelens et al. (33), Arbeit (3) and Maslow et al. (22). The performance criteria include the stability, discriminatory power, and reproducibility of the typing system.

MATERIALS AND METHODS

Bacteria. Strains of *S. aureus* (n = 463) were pooled from eleven collections previously used for several purposes (Table 1). For cultivation, bacteria from glycerol stocks, stored at -80°C, were inoculated on Columbia III agar (Becton Dickinson, Etten-Leur, The Netherlands) supplemented with 5% sheep blood and incubated at 37°C for 24 h. All strains were identified as *S. aureus* by standard microbiological methods (19). Methicillin resistance was determined by "direct-colony suspension" inoculation of the strains on Mueller Hinton agar (Oxoid CM 337, Brunswig, Amsterdam, The Netherlands) in the presence of a disk containing 5 µg of methicillin (Oxoid; Brunswig, Amsterdam, The Netherlands). And after 16 to 18 h of incubation at 35°C, zone diameters were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (26).

Table 1. Characterization of the *Staphylococcus aureus* collections used in this study[a].

Collection	Collection code(s)[a]	Geographic origin[b]	No. of strains	Original purpose for strain collection in this study	Description of the collections	Reference(s) of source
1.		United States (CDC)	59		Multicenter collection of MRSA (63%) and MSSA (37%) strains.	35
2.		Portugal	184		Nationwide disseminated MRSA strains from hospitals	1
3.		Worldwide	66	Generation of strain-specific DNA probes	Worldwide collection of MRSA strains	20
4.		Italy	49		Genetically unrelated MRSA strains (determined by PFGE), isolated from hospitalized patients (five centers), Sicily, Italy	41
5.		Australia	18		Genetically unrelated MRSA strains (determined by PFGE), obtained from four different hospitals	41
6.	K2	United States	26	Determination of discriminatory power of the genotyping methods (see Table 4)	Community-acquired MRSA strains	46
7.	SA, SB, SC	United States (CDC)	14		Selection of geographically diverse strains from collection 1	35
8.	NC	The Netherlands	10	Stability experiment for binary typing probes (see Table 3)	Strains isolated from persistent nasal carriers	43
9.	RIVM	The Netherlands	2		Selection of 1 MRSA strain (Va) and 1 MSSA strain (Ia), from collection no. 10	
10.	RIVM	The Netherlands	49	Epidemiological applications of the diverse genotyping systems (see Table 5)	MRSA and MSSA strains from 10 outbreaks in Dutch hospitals	This study
11.		United Kingdom (NCTC)	2	Mapping of the strain-specific DNA probes (see Fig. 1)	*S. aureus* NCTC 8325 and 8325-4	27, 29

[a] K2, community-acquired MRSA strains from a New York City hospital; SA, SB, SC, *S. aureus* strains from the CDC collection; NC, nasal carrier; RIVM, National Institute of Public Health and the Environment (Bilthoven, The Netherlands).
[b] CDC, Centers for Disease Control and Prevention; NCTC, National Collection of Type Cultures (Central Public Health Laboratory, London, United Kingdom).

Binary typing. Binary typing was performed as described previously by van Leeuwen et al. (45, 46). However, we increased the overall number of strain-specific DNA probes from 5 to 15. The same procedures were used for the generation of the DNA probes as described before (45).

(i) Generation of the strain-specific DNA probes. In short, after RAPD analysis DNA fingerprints were compared visually, and unique, strain-differentiating amplicons were selected and subsequently cloned into the TA cloning vector (Invitrogen, Leek, The Netherlands) and then transformed into *Escherichia coli* JM 109 cells. Inserts were amplified from the recombinant plasmids with M13 and T7

primers. Cloned fragments were characterized by DNA sequencing with dye-terminator chemistry by using a 373 DNA sequencing system (Perkin-Elmer, Foster City, Calif.). The insert sequences were compared with all entries of the databank of the National Center for Biotechnology Information (NCBI) and were analyzed for nucleotide and protein sequence similarities with the Basic Local Alignment Search Tool (BLASTN and BLASTP, respectively [2]).

(ii) Implementation of the binary typing system. Labeling, hybridization, and detection of the cloned DNA fragments were performed with enhanced chemiluminescence (ECL) direct labeling and detection systems, according to manufacturer's protocols (Amersham Life Science, Buckinghamshire, United Kingdom), in order to use them as probes. The hybridization characteristics of the DNA probes were defined by prescreening these probes on a Southern blot containing 14 genetically unrelated staphylococcal strains (Table 1, collection 7). DNA probes displaying differential hybridization were added to the binary typing system. Hybridization of the 15 different DNA probes was scored with a 1 or a 0 according to the presence or absence of the hybridization signal, respectively, and the resulting binary code was transformed into a decimal number. This number is further represented as the binary type.

RAPD analysis. RAPD analysis was carried out essentially as described before (37). Fingerprints were scored visually in which a single band difference defined a novel RAPD type. The three-letter codes are based on ERIC-2, AP-1 and AP-7 priming (45) and can only be compared within each group and not across the different groups of organisms represented in Tables 3, 4 and 5.

PFGE. Restriction with *Sma*1 (Boehringer Mannheim, Germany) of genomic staphylococcal DNA and subsequent separation of the DNA macrorestriction fragments was performed by contour-clamped homogeneous electric field (CHEF) PFGE as described before (39). Macrorestriction profiles were interpreted as described by Tenover et al. (36), and each pattern is presented as a roman letter.

***MecA*-Tn*554* probe typing.** Genomic staphylococcal DNA was digested with *Cla*1 endonuclease (Pharmacia Biotech, Roosendaal, The Netherlands) according to the manufacturer's instructions. Generation of target-specific probes and hybridization was done as described before (20).

Coagulase gene PCR. Coagulase gene polymorphism was determined by PCR as described previously (32). The amplified part of the coagulase gene was digested with the restriction endonuclease *Alu*1 (Boehringer) according to the manufacturer's protocol. Restriction fragment length polymorphism (RFLP) patterns were visually interpreted and indexed by roman lettering.

Table 2. Summary of the demographic data and the sequence homologies from the RAPD-generated *S. aureus* DNA fragments with strain-specific characteristics (n=15).

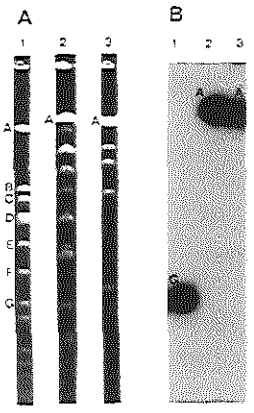
Probe code	Origin	Source strain code	MC result[b]	Probe size (bp)	specificity[c]	Nucleotide sequence homology[d]		Protein sequence homology[d]	
						M13	T7	M13	T7
AW-1	CDC[a]	SA-08	R	1200	13	NH	NH	NH	NH
AW-2	CDC	SA-01	R	600	8	NH	NH	NH	NH
AW-3	CDC	SA-06	I	550	7	NH	NH	HrmA	NH
AW-4	CDC	SA-02	R	700	6	*S. aureus* plasmid pSH6	*S. aureus* plasmid pSH6	*S. aureus* transposase IS257	*S. aureus* transposase IS257
AW-5	Portugal	HPV107	R	400	3	NH	NH	Lysostaphin precursor of *S. simulans*	NH
AW-6	United States	BK1591	R	350	5	NH	NH	NH	NH
AW-7	Italy	246D	R	1200	4	NH	NH	NH	NH
AW-8	Australia	WBG8217	R	350	5	*B. subtilis yqeV*	*B. subtilis yqeV*	*B. subtilis* hypothetical protein YqeV	*B. subtilis* hypothetical protein YqeV
AW-9	United States	BK1457	R	1500	5	NH	NH	NH	NH
AW-10	United States	BK1461	R	400	6	NH	NH	NH	NH
AW-11	Australia	WBG8231	R	1500	6	NH	NH	NH	NH
AW-12	CDC	SB-18	R	350	4	NH	NH	NH	NH
AW-13	Italy	85CCH	R	350	4	NH	NH	NH	NH
AW-14	Unites States	BK1563	R	650	4	NH	NH	NH	NH
AW-15	Italy	76CCH	R	300	5	NH	NH	NH	NH

[a] CDC, Centers for Disease Control and Prevention

[b] Mc; methicillin susceptibility disk-diffusion test; R, resistant; I, intermediate.

[c] Number of strains hybridizing with a particular probe. The probes were tested on 14 CDC strains (Table 1, collection nr. 7).

[d] NH, no homology. Cloned DNA sequences of insert termini (M13 and T7), analyzed for sequence homologies with nucleotide and protein sequences in the NCBI by using the BLAST computer program (2).



DNA probe	Hybridization to fragment		
	Sma-1	*SgrA1*	*Asc-1*
AW-1	NH	NH	NH
AW-2	NH	NH	NH
AW-3	C	A	A
AW-4	NH	NH	NH
AW-5	C	A	C
AW-6	A	F	D
AW-7	NH	NH	NH
AW-8	A	H	E
AW-9	B	C	B
AW-10	A	F	D
AW-11	E	A	A
AW-12	A	F	D
AW-13	A	F	D
AW-14	A	F	D
AW-15	G	A	A

Figure 1.Physical mapping of the DNA probes on *Staphylococcus aureus* NCTC 8325 (29) restriction fragments. (A) PFGE macro-restriction patterns of *S. aureus* NCTC 8325 digested with *Sma*I, *Sgr*A1, *Asc*I (lanes 1, 2, 3 respectively). Restriction fragments are coded by descending molecular size. (B) Example of hybridization results using probe AW-15 to PFGE patterns of *S. aureus* NCTC 8325. Lanes are the same as for panel A. (C) Hybridization results of probe AW-15 depicted on the physical map of *S. aureus* NCTC 8325. (D) Mapping results of the 15 strain-specific DNA probes (AW-1 through AW-15) to the macro-restriction fragments of the *S. aureus* NCTC 8325 genome. NH, no hybridization of the strain-specific DNA probe to the macrorestriction fragments.

PCR analysis of the *mec* regulator genes *mecI* and *mecR1*. PCR was performed as described before (34). Three sets of specific primers were used to amplify the different regions of the *mec* regulator genes, i.e. *mecI* and the 5'- end (transmembrane part) and the 3'-end (penicillin-binding part) of *mecR1*.

***cna* probe.** The presence or absence of the *S. aureus* collagen adhesin (*cna*) was used as an additional genotypic marker for the differentiation of *S. aureus* strains. Probing was performed essentially as described by Smeltzer et al. (31).

***spa* gene.** Staphylococcal protein A (Spa) gene polymorphism was determined by PCR as described previously (11). The so-called X-region, a repetitive part within the gene, was amplified, and subsequently, the amplicon was digested with the restriction endonuclease *Rsa*I (Boehringer) resulting in two fragments composed of 214 and 35 bases and a third fragment containing the repetitive DNA. The number of 24-bp repeats was calculated by comparison with a 100-bp molecular weight marker (Pharmacia Biotech).

Phage typing. Phage typing was performed at the Dutch National Institute of Public Health and the Environment by using the international set of typing phages and a set of typical Dutch phages (28, 47). Different phage patterns were given different type designations.

Ribotyping. Conventional ribotyping with *Eco*R1 was performed by methods

described previously (13). Restriction fragments were Southern blotted onto Hybond N$^+$ membranes (Amersham) (30), and the *S. aureus* 16S rRNA gene, amplified by PCR, was used as a probe. Hybridization was detected by using an ECL kit (Amersham).

***MecA* PCR.** All *S. aureus* strains were investigated for the presence of the *mecA* gene by PCR as described before (24).

Physical mapping. Genomic DNAs from *S. aureus* NCTC 8325 (29) and 8325-4 (27) was digested with *Sma*I (Boehringer), *Sgr*A1 (Boerhringer), and *Asc*I (New England Biolabs, Leusden, The Netherlands) according to the manufacturer's protocols. Macrorestriction fragments were separated by PFGE and subsequently transferred onto Hybond N$^+$ membranes (Amersham) for Southern hybridization (30). Probing with the 15 strain-specific DNA fragments was done as described above under "Binary typing".

Statistical analysis. The discriminatory power of binary typing and other genotyping formats used in this study, defined as the average probability that different genotypes will be assigned to two unrelated strains in the population of a given genus, was calculated by using the formula of the Simpson index of diversity as explained by Hunter and Gaston (17, 18). The contribution of the DNA probes to the discriminatory power of the binary typing system was analyzed by cluster analysis and comparison of the dendrograms. First, all of the probes (n=15) were used to characterize 40 unique (Table 1, collection no. 6 and 7) and 10 outbreak clusters (Table 1, collection no. 10) of *S. aureus* strains. The percentages of similarity of the hybridization patterns were calculated with Dice coefficient and with unweighted pair group mathematical analysis (UPGMA) to display relatedness hierarchies among the strains. Subsequently, the procedure was repeated after discarding the DNA probe that had the lowest level of discrimination.

RESULTS

Selection of the strain-specific DNA probes. RAPD analysis with multiple primers was performed on 376 *S. aureus* strains (Table 1, collections 1 through 5) of which 97% was methicillin-resistant. One hundred and twenty-four amplicons were selected from 66 RAPD patterns. Overall, 98 DNA fragments (79%) were successfully cloned, and from those a total number of 17 clones displayed a strain-specific character after hybridization with *Eco*R1 digested DNA from the 14 epidemiologically unrelated *S. aureus* strains (Table 1, collection 7 [38% MRSA]).

Table 3. Stability of the strain-differentiating DNA probes determined with *S. aureus* strains obtained from persistent nasal carriers.

Persistent carrier (sex)[a]	Strain[b]	Isolation date	Binary code (type)[c]	Results by indicated genotyping technique[d]: RAPD analysis	PFGE	*spa* polymorphism	Coagulase gene polymorphism	Presence / absence of *cna*
099 (F)	NC-711	1988	001010110101111 (5551)	CCC	J	9	F	-
	NC-220	September 1995	001010110101111 (5551)	CCC	J	9	F	-
060 (F)	NC-1740	1988	101010011100101 (21733)	FCC	M	9	D	-
	NC-1733	February 1995	101010011100101 (21733)	FCC	M	5	D	-
038 (F)	NC-1288	1988	000000011100111 (231)	EDE	G	7	B	-
	NC-105	June 1995	000000011100111 (231)	EDE	G	7	B	-
076 (F)	NC-705	1988	000010011100101 (1253)	AEA	A	7	A	+
	NC-054	May 1995	000010011100101 (1253)	AEA	A	7	A	+
145 (M)	NC-714	1988	000000010100101 (165)	BBB	B	8	B	+
	NC-063	May 1995	000000010100101 (165)	BBB	B	8	B	+

[a] Persistency defined as 10 identical culture results from longitudinally sampling over 3 months (43).
[b] NC, strain from nasal carrier. See Table 1, collection 8, for more details.
[c] Overall results after hybridization with 15 strain-specific DNA probes (AW-1 through AW-15, respectively). The decimal number represents the binary type. See Materials and Methods for more information.
[d] Results (except for presence / absence of *cna*) are given as codes for each technique. See Material and Methods for more information.

However, 3 of the 17 clones shared the same DNA sequence, and two of these were consequently discarded. The remaining 80 fragments hybridized with DNA of all strains either at single (n=42) or multiple sites (n=14) or recognized the digested DNA from their source strain (n=24) only. The latter fragments were not included since these fragments did not contribute significantly to the discriminatory power of the system.

Characterization of the DNA probes. The origin and the nature of the 15 RAPD-generated DNA probes are outlined in Table 2. Sequence data were obtained from both termini (M13 and T7), and the DNA sequences were analyzed separately for homology by using the BLAST program with the nucleotide and protein sequence data bank, including the unfinished microbial genomes data bank (NCBI). A large proportion of these sequences did not match with known DNA elements (87% for the nucleotide sequence data bank and 80% for the protein data bank). Probe AW-3 (M13 terminus) appeared to have a low score (BLAST score of 36) with the gene product encoded by *hrmA* of *Nostoc sp.* in the protein sequence database. Probe AW-4 (both termini) displays a high score (1571) with the *S. aureus* multiresistance plasmid pSH6 for insertion sequences IS256 and IS257 in the search of the nucleotide sequence data bank and a high score (593) with IS257 transposase in the search for the protein sequence data bank. The M13 terminus of probe AW-5 displayed a low homology score (89) with the lysostaphin precursor of *Staphylococcus simulans*. Finally, probe AW-8 (both termini) appeared to have a high level of similarity (BLAST score 573) with the *yqeV* gene, a hypothetical protein, and part of the polycystronic locus of the *Bacillus subtilis dnaK* operon.

The locations of the strain-specific DNA probes were determined on the physical map of the *S. aureus* NCTC 8325 genome (Fig. 1) and on the restriction fragments of *S. aureus* NCTC 8325-4. Four of the 15 DNA probes (AW-1, AW-2, AW-4, and AW-7) failed to hybridize to either of the two staphylococcal genomes. Five probes (AW-6, AW-10, AW-12, AW-13, and AW-14) were found to be physically clustered in the same DNA region (position 2500 to 2600 kb), while the remaining seven probes were found to be scattered on the physical map of *S. aureus* NCTC 8325.

Stability experiments. The in vivo stability of the binary typing system was assessed by testing sequential isolates of *S. aureus* from five individuals who were previously classified as persistent nasal carriers (Table 1, collection 8) (43). The 15 DNA probes uniformly and correctly identified each of the two *S. aureus* strains isolated from five persistent nasal carriers in 1988 and 1995, respectively, in accordance with the other genotyping techniques (Table 3). Moreover, we tested the in vitro stability of the DNA probes by serial passage (50x) of strains Ia and Va (Table 1, collection 9). Again, all descendent isolates were shown to be identical i.e., their binary types did not change with serial passages (data not shown).

Table 4. Analysis of the discriminatory power of the binary typing method compared with other genotyping techniques, estimated on the typing results of epidemiologically unrelated MRSA strains from New York City and geographically diverse *S. aureus* strains from the United States.

strain	binary code	binary type	Result by indicated genotyping technique			
			PFGE	RAPD analyses	*mecA*/Tn*554* probing	Methicillin resistance[b]
K2-01	001110111110111	7671	J	HMC	IV:M	R
K2-02	110001011100101	25317	K	AAK	II:F	R
K2-06	001110111100111	7655	L	NHB	III:NH	R
K2-07	000100100100111	2343	M	CCC	II:NH	R
K2-12	001110100100111	7463	J	HMC	IV:M	R
K2-13	101110111110101	24053	M	NBB	II:NH	R
K2-19	001100100100101	6437	J	MMC	IV:M	R
K2-20	011011111100001	14305	N	NHB	II:NH	R
K2-21	111110011100001	31969	O	NBB	II:NH	R
K2-22	000010111111111	1535	P	BBB	I:unique	R
K2-24	001100000110111	6199	Q	NNA	I:NH	R
K2-30	001110100110011	7475	J	GOC	I:A	R
K2-31	011111110111101	16317	R	NHB	II:NH	R
K2-32	001110100111111	7487	J	HMC	IV:M	R
K2-34	011110111111101	15869	S	NHB	II:NH	R
K2-38	011110111110101	15861	T	BIB	I:A	R
K2-40	011110111110001	15857	T	BBB	I:A	R
K2-44	001100101110011	6515	U	MNA	I:E	R
K2-45	001110001100011	7267	J	HMC	IV:M	R
K2-47	011110011100001	15585	V	NHB	II:NH	R
K2-50	001111111101111	8175	V	BBB	I:A	R
K2-51	101111111111101	24573	V	BBB	I:A	R
K2-52	001111010110111	7863	J	HMC	IV:M	R
K2-56	011110011110011	15603	W	HMC	unique:M	R
K2-57	000100110111101	2493	X	JAE	I:D	R
K2-65	101110110100011	23971	Y	PPH	unique:E	R
SA-04	111010000000000	29696	E	FSL	NH:X	S
SA-06	101110000100000	23584	C	TTM	II:NH	I
SA-07	111110011100000	31968	B	TTM	NH:NH	S
SA-08	111110011100001	31969	G	VSL_1	I:NH	R
SA-11	100100001000000	18496	F	VSL_1	II:NH	R
SA-12	111110001100000	31840	A	TTM	I:A	R
SA-13	111110000110000	31792	A	TTM	I:A	R
SA-14	100100000100000	18464	H	TTM_1	NH:NH	S
SA-17	111110000010000	31760	A	TTM	I:A	R
SB-02	000001101011111	863	I	WUN	NH:NH	S
SB-09	111101110011111	31647	I	WVN	NH:Z	S
SB-13	111101110011111	31647	I	WVN	NH:NH	S
SC-06	101111110000111	24455	Z	JXO	NH:NH	S
SC-08	100000000011000	16408	Z	JXO	NH:NH	S
Total no. of types		38	25	19	11[c]	3
D		0.998	0.966	0.949	0.848	

[a] See Table 1 for more information on the origins of the strains used.
[b] R, resistant; I, intermediately resistant; S, susceptible
[c] The *mecA*/Tn*554* typing data of the MSSA strains (n=8) were deleted.

Discriminatory power. We compared the discriminatory power of our binary typing system with that of generally accepted typing systems included PFGE, RAPD analysis, and *mecA*-Tn*554* probing. Comparative analysis of the discriminatory power of these genotyping systems is displayed in Table 4 and is expressed by the Simpson index of diversity (*D*). RFLP analysis of the *mecA* gene and Tn*554* generated 11 unique patterns from the 40 epidemiologically unrelated strains (Table 1, collections 6 and 7) and had the lowest score ($D = 0.848$). Due to the absence of the *mecA* gene, the typing data of the MSSA strains were deleted for the *D* value determination. PFGE and RAPD differentiated the collection into 25 and 19 subtypes with *D*-values of 0.966 and 0.949, respectively. However, the binary typing system distinguished 38 unique genotypes and had a *D* score of 0.998. Only two binary types, 31969 and 31647, were each found twice in the collection. Type 31647 was found to be identical by PFGE, RAPD and *mecA* gene polymorphism, but one strain (SB-13) lacked Tn*554*. Types 31969 and 31647 reportedly also share a single phagetype (35). Strains K2-21 and SA-08 share binary type 31969 but clearly differed by the other genotyping systems (Table 4). It has to be emphasized that a common clonal origin for some of the (even epidemiologically unrelated) strains described in the present communication cannot be fully excluded.

Reproducibility. In order to test the reproducibility of our binary typing system, we tested 10 different clusters of epidemiologically related *S. aureus* strains (four to five strains per outbreak; four MSSA and six MRSA clusters). A representative illustration of binary typing hybridization results is outlined in Fig. 2. The genetic relatedness of the strains within a cluster was primarily defined on the basis of epidemiological data and possession of identical phage types within the cluster. The reproducibility of the binary typing technique was calculated as the number of isolates correctly assigned to the same type within a cluster divided by the total number of strains tested. Overall 45 of 49 (92%) strains were correctly typed, i.e., 30 of 30 MRSA and 15 of 19 MSSA strains (Table 5). Interestingly, the nonconcordant MSSA strains also showed genetic variation by one or more of the other genotyping systems applied to the same set of strains. Thus, binary typing is similarly sensitive to such variation in the genome of *S. aureus*.

The informative value of the DNA probes. The contribution of the DNA probes to the discriminatory power of the system was analyzed by comparison of the dendrograms. Increasing substraction of the distinct DNA probes reduced the resolution of the binary typing system among the genotypic results of the unique *S. aureus* strains (Fig. 3a). A similar effect on the epidemiological concordance among the hybridization patterns of the *S. aureus* outbreak cluster strains was noticed after substraction of the same DNA probes (Fig. 3b). Consequently, none of the probes can be discarded from the binary typing system.

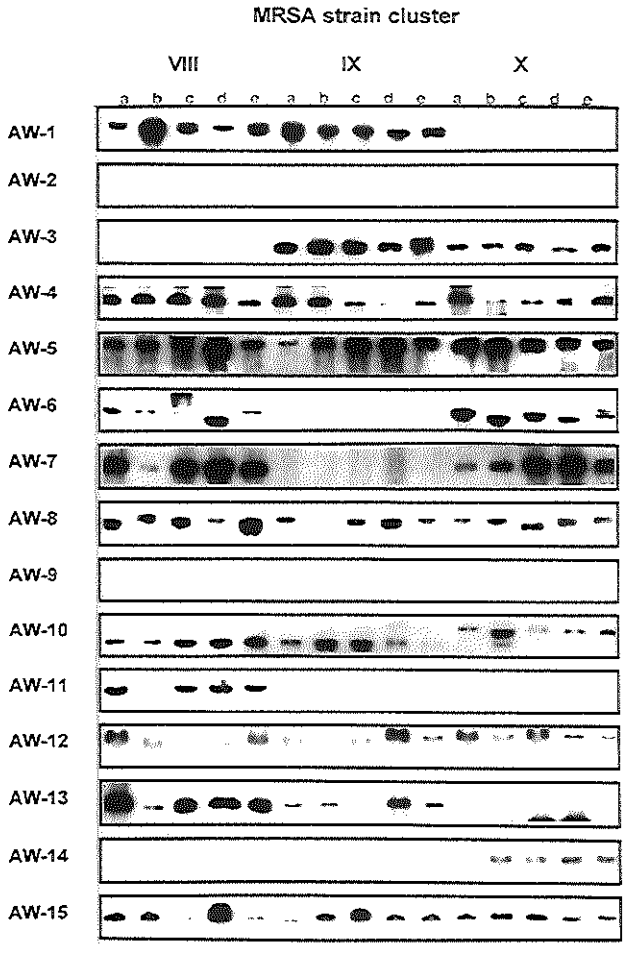


Figure 2. Representative example of binary typing results, obtained with the complete panel of strain-specific DNA probes (AW-1 through AW-15) from three MRSA clusters VIII, IX and X (n=15, collection 10).

DISCUSSION

Whole genomes of bacteria are currently being sequenced at high rates, and information can be derived from analysis and comparison of these chromosomes. Essential paralogous regions as well as narrowly distributed gene families can be identified. The latter group may be genus, species, or even strain specific. For instance, the genome of *Mycoplasma genitalium* commits about 5% of its content to a single species-specific domain, encoding an adhesin gene (10). Another type of DNA variability was observed after the completion of the *Haemophilus influenzae* DNA sequence (9). Repeats in the genes encoding enzymes involved in lipopolysaccharide biosynthesis and iron acquisition and a gene encoding an adhesin display clear heterogeneity (16, 40). The *E. coli* genome highlights novel insertion sequence

elements, phage remnants, and many DNA fragments of unusual composition, indicating genome plasticity and horizontal gene transfer (5). Many bacterial virulence genes are found as discrete DNA fragments, present in pathogenic organisms but absent from nonpathogenic members of the same genus or species, e.g., the "pathogenicity islands" of uropathogenic *E. coli* or enteropathogenic *E. coli* (14, 23). Unfortunately, only a single genome sequence of a grampositive bacterium is known. The *B. subtilis* genome contains phage-type elements as well, again indicating DNA flexibility (21). Based on theoretical comparative analysis, many DNA elements contributing to DNA variation can be pinpointed. No experimental studies has been described as yet, however. Practically, the genome variability of *S. aureus* strains can be visualized on the basis of RAPD analysis and the use of the amplicons thereof as probes. We describe here an approach for isolating species-specific DNA elements for a bacterium for which the whole genome sequence is not in the public domain.

The aim of the present study was to validate the use of strain-differentiating DNA probes for the genotyping of *S. aureus* and to develop a new typing format, providing a simple binary output based on the use of RAPD-generated DNA probes. Such probes can detect sequence variation between genomes without prior knowledge of the target DNA sequence, as has been presented before (45, 46). We now have extend the number of DNA probes to 15 and have shown the typing system to have a very high index of reproducibility, stability (100%) and discriminatory power (D = 0.998). Hybridization studies revealed that only 12% of the RAPD amplicons, visually selected for uniqueness, exhibited the desired genetically typing characteristics for *S. aureus* strains. Primer site variation may be the origin of the remaining 88% differentiating amplicons. The nature of the DNA probes used in this study remains largely unknown. In one case (probe AW-4) homology with a mobile genetic element, IS257, was found. IS257 is an insertion sequence identified as commonly occurring in staphylococcal plasmids (7). These plasmids often code for diverse resistance determinants. The investigation of further alignments awaits publication of the whole *S. aureus* genome sequence.

The locations of the DNA probes on the physical map of *S. aureus* NCTC 8325 (29) (Fig. 1) and *S. aureus* NCTC 8325-4, a derivative of 8325 cured of phages P11, P12 and P13, (27), were determined. Some probes (n = 4) were neither on the physical map of *S. aureus* NCTC 8325 nor present on the restriction fragments of NCTC 8325-4. The remainder of the probes recognized elements on both genomes, which argues against a putative relationship with the prophage sequences that are present in *S. aureus* NCTC 8325 but not in *S. aureus* NCTC 8325-4. Seven probes showed random location and five clustered together around the 2500- to 2600-kb region of the *S. aureus* NCTC 8325 genome. These latter probes all share a nucleotide sequence of 80 bp, but the main part of their nucleotide sequence was different. It is possible that this DNA region is part of a direct repeat and spacer region, which can be used to generate sequence variation patterns between genomes (44). The location of these probes coincides with that of several potentially variable elements: essential genes for recombination between genomes (*recA*) or DNA repair (*uvr*), virulence

factors (*hla*), and diverse Tn*551* insertion-sites (29). The probes that hybridized to DNA regions scattered throughout the genome seemed to have no linkage with variable DNA sequences, except for one probe (AW-15) which is located in the vicinity of resistance determinants (*mec* region), virulence factors (*spa*) and the origin of replication.

The stability of the binary typing system was evaluated with sequential isolates recovered from healthy individuals who were shown to persistent nasal carriers of *S. aureus* (43). The persistent carriers were monitored in 1988 and 1995, and similarity of the genotypes among these two sampling periods were determined with binary typing, PFGE, RAPD analysis, coagulase and protein A gene polymorphism and the absence or presence of the *cna* gene. All genomic characterization techniques (Table 3), including the 15 epidemiological markers of the binary typing system, indicated a high degree of genomic stability over the years, except for the *spa* gene typing (persistent carrier 060). During laboratory storage and replication, mutations and transpositional recombination may occur (6), and the stability of the epidemiological markers for the staphylococcal genome can be measured by the in vitro stability. The in vitro stability of the binary typing system was estimated by comparing the genomes of strains before and after 50 serial passages of strains on culture media. All DNA probes generated identical results after repeated testing (data not shown).

The Simpson index of diversity (*D*) expresses the discriminatory power of a genotyping system (17, 18). We calculated the *D* value for binary typing and compared this with the results of other frequently used techniques (Table 4, PFGE, RAPD analysis, *mecA* - Tn*554* probing). Certain systems, such as PFGE or RAPD analysis with multiple primers, generate complex banding patterns, and the Simpson index was calculated on the basis of the similarity level, defining a genotype (36). Hunter (18) proposed that the standardized discrimination index determines the discrimination index of a typing method that has a reproducibility of 95%; this is designated D_{95}. Both binary typing and PFGE exceed the level of D_{95}, and consequently these methods can be used as a single method. Less discriminating systems such as RAPD analysis and *mecA* - Tn*554* probing can be used in combination to obtain a significant D_{95} index (18, 33).

The probability of clonal linkage among epidemic strains determined to be similar by diverse genotyping techniques can be expressed at the level of reproducibility. In fact an application of in vivo stability, i.e., comparison of sequential isolates, recovered along the course of a well-documented outbreak (33).

Table 5. Survey of geno- and phenotypic results for epidemic outbreak strains of MRSA and MSSA from Dutch hospitals and nursing-homes.

Cluster code	Epidemiological data: Hospital	Patient	Isolate	Isolation date (mo/yr)	Binary code[b]	Binary type[c]	Phage type[d]	Ribo type[e]	Results for indicated typing method: PFGE[f]	RAPD[g]	coagulase gene polymorphism[h]	*cna*[i]	*spa*[j]	*mecA*[k]	*mecI*[k]	*mecR*[k] 5'	*mecR*[k] 3'
MSSA																	
I a	A	1	groin	11-96	101001111111101	21501		A	A	AAA	A	-/-	11	-	-	+	-
I b	A	1	sputum	11-96	101001111111101	21501		A	A	AAA	A	-/-	11	-	-	+	-
I c	A	1	blood	11-96	101001111111101	21501		A	A	AAA	A	-/-	11	-	-	+	-
I d	A	2	sputum	11-96	101001111111101	21501		A	A	AAA	A	-/-	11	-	-	+	-
I e	A	3	blood	07-96	101001111111101	21501		A	A	AAA	A	-/-	11	-	-	+	-
II a	B2	1	pus	12-96	001000000100000	4128		A	B	BBB	NR	+/+	5	-	-	+	-
II b	B1	1	pus	10-96	001000000100000	4128		A	B	B_1BB	NR	+/+	5	-	-	+	-
II c	B1	2	pus	08-96	001000000100000	4128		A	B_1	B_1BB	NR	+/+	10	-	-	+	-
II d	B2	3	pus	11-96	001000010100000	4256		A	B_2	B_1BB	NR	+/+	11	-	-	+	-
II e	B2	4	pus	10-96	001000000100000	4128		A	B	BBB	NR	+/+	5	-	-	+	-
IIIa	B1	5	pus	11-96	000000110101011	427		B	C	CCC	NR	-/-	10	-	-	+	-
III b	B3	6	urine	10-96	000000110101011	427		B	C	CCC	NR	-/-	10	-	-	+	-
III c	B3	6	urine	10-96	000000110101011	427		B	C	CCC	NR	-/-	10	-	-	+	-
III d	B3 (B1)[l]	7	pus	12-95	010000110101011	8619		B	C_1	CCC	NR	-/-	10	-	-	+	-
III e	B1	8	urine	02-96	010000110101011	8619		B	C_1	CCC	NR	-/-	10	-	-	+	-
IV a	C	1	nose	11-96	001011010100010	5794		A	D	DDD	B	-/+	7	-	-	+	-
IV b	D (C)[m]	2	exit site	04-96	001001010100010	4770		A	D	DDD	B	+/+	9	-	-	+	-
IV c	C	3	nose	07-96	001011010100010	5794		A	D_1	DDD_1	B	-/+	11	-	-	+	-
IV d	C	4	pus	08-96	001011010100010	5794		A	D_1	DDD_1	B	-/+	11	-	-	+	-

table to be continued on next page

MRSA																	
V a	E°	1	unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
V b	E	2	unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
V c	E	3	unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
V d	E	4	unknown	01-96	110111111111111	28671	XVI-3	A	E	EEE_1	C	+/+	10	+	+	+	+
V e	E	5	unknown	01-96	110111111111111	28671	XVI-3	A	E_1	EEE_1	C	+/+	10	+	+	+	+
VI a	F1°	1	unknown	10-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	-	+	-
VI b	F1	2	unknown	05-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	-	+	-
VI c	F1	3	nose	05-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	-	+	-
VI d	F1	4	unknown	07-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	-	+	-
VI e	F1	5	unknown	04-96	001110010000000	7296	XVI-4	A	F	FFB	NR	+/+	9	+	-	+	-
VII a	F2°	1	nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	-	+	-
VII b	F2	2	nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	-	+	-
VII c	F2	3	nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	-	+	-
VII d	F2	4	nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	-	+	-
VII e	F2	5	nose	10-96	001110000000000	7168	XI-6	A	F_1	FFB	NR	+/+	9	+	-	+	-
VIII a	G	1	sputum	01-96	100111110111101	20413	Z-72	A	G	GGF	D	-/-	10	+	-	+	-
VIII b	G	2	unknown	03-96	100111110111101	20413	Z-72	A	G	GGF	D	-/-	10	+	-	+	-
VIII c	G	3	unknown	04-96	100111110111101	20413	Z-72	A	G	GGF	D	-/-	10	+	-	+	-
VIII d	G	4	pus	04-96	100111110111101	20413	Z-72	A	G	GGF	D	-/-	10	+	-	+	-
VIII e	G	5	nose	04-96	100111110111101	20413	Z-72	A	G	GGF	D	-/-	10	+	-	+	-
IX a	H	1	urine	01-96	101110010101101	23725	III-29	A	H	HAA	D	-/-	9	+	-	+	-
IX b	H	2	urine	01-96	101110010101101	23725	III-29	A	H	HAA	D	-/-	9	+	-	+	-
IX c	H	3	pus	01-96	101110010101101	23725	III-29	A	H	HAA	D	-/-	8	+	-	+	-
IX d	H	4	pus	01-96	101110010101101	23725	III-29	A	H	HAA	D	-/-	9	+	-	+	-
IX e	H	5	pus	01-96	101110010101101	23725	III-29	A	H	HAA	D	-/-	9	+	-	+	-

table to be continued on next page

X 1	I	1	ven. line	01-93	001111110101011	8107	III-29	A	I	IAA	D	-/-	10	+	-	+	-
X 2	I	2	nose	01-93	001111110101011	8107	III-29	A	I	IAA	D	-/-	10	+	-	+	-
X 3	I	3	wound	02-93	001111110101011	8107	III-29	A	I	IAA	D	-/-	10	+	-	+	-
X 4	I	4	bile	02-93	001111110101011	8107	III-29	A	I	IAA	D	-/-	10	+	-	+	-
X 5	I	5	wound	02-93	001111110101011	8107	III-29	A	I	IAA	D	-/-	10	+	-	+	-

[a] See Table 1, collection 10 for more details.

[b] Overall results after hybridization with 15 strain-specific DNA probes (AW-1 through AW-15, respectively).

[c] Binary code transformed into a decimal number.

[d] MSSA strains have identical phage types within a cluster. Types were dropped on account of the extensive code.

[e] Results with *Eco*R1 digested genomic DNA.

[f] After *Sma*1 macro-restriction analysis.

[g] The three letter code summarizes the typing results per primer used (first letter, primer ERIC-2; second letter, primer AP-1; third letter, primer AP-7).

[h] RFLP analysis of the coagulase gene and subsequent digestion of the amplicon with *Alu*1. NR, no restriction sites on the amplicon.

[i] PCR result with *cna* gene-specific primers is presented before the shill, and the plus or minus after the shill describes the hybridization result with the PCR generated, *cna*-specific DNA probe.

[j] Analysis of the so-called X-region by PCR. Amplicons were digested with *Rsa*1. The number of direct repeats in this region was determined after electrophoresis.

[k] -, absence; +, presence of the *mec*-specific DNA regions analyzed by PCR.

[l] Patient removed from hospital B3 to an annex hospital B1.

[m] Patient was transferred from nursing home D to hospital C.

[n] Outbreak in a hospital on the Netherlands Antilles.

[o] F1 and F2 are subdivisions in different locations of the same hospital.

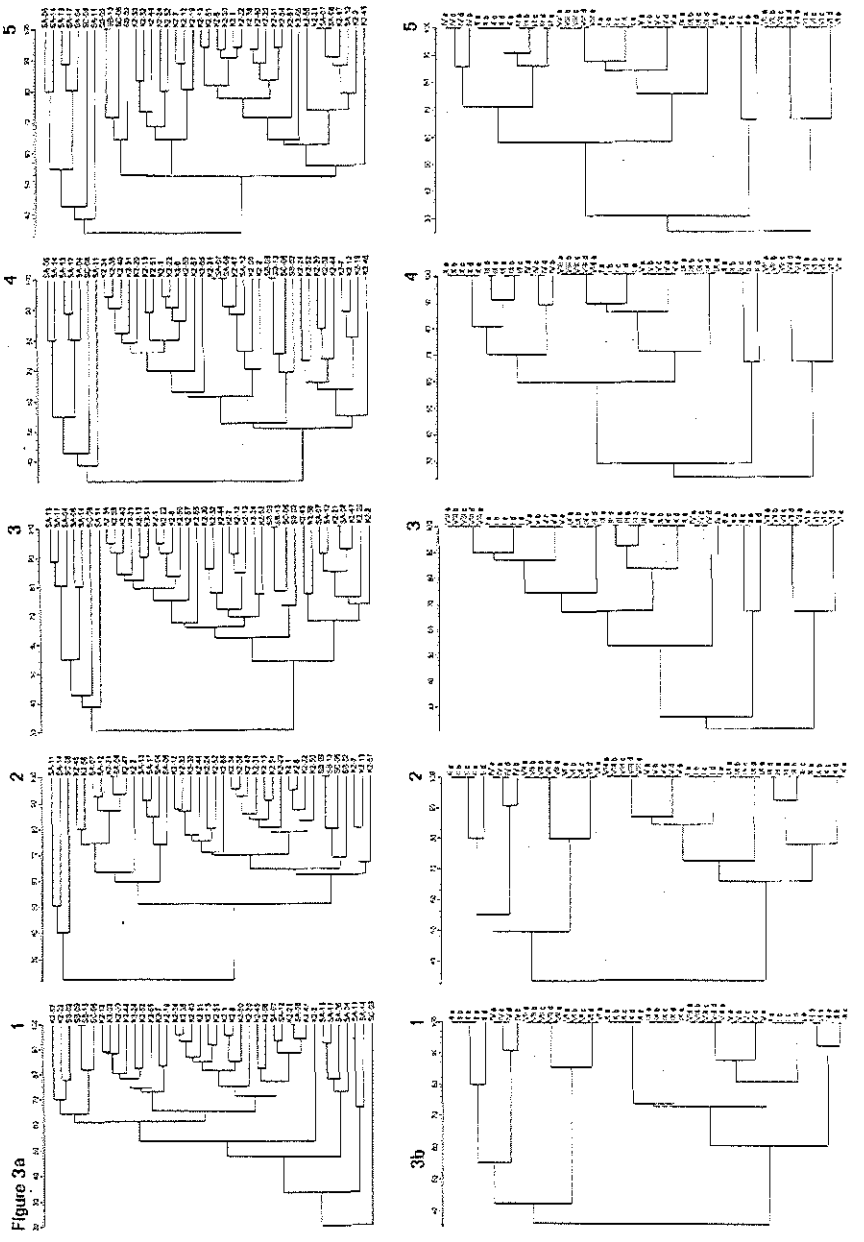


Figure 3. (A) Dendrogram displaying the grouping of 40 unique *S. aureus* strains (collection nr. 6 and 7, Table 1) on the basis of hybridization scores after binary typing with all DNA probes (1); deletion of probe AW-4 (2); deletion of probes AW-4 and AW-3 (3); deletion of probes AW-4, AW-3 and AW-15 (4); deletion of probes AW-4, AW-3, AW-15 and AW-6. (B) Dendrogram presents the similarity percentage of the hybridization patterns of 10 outbreak clusters of *S. aureus* strains (collection nr. 10, Table 1), obtained with the complete panel of DNA probes comprising the binary typing system (1); after deletion of probe AW-4 (2); after deletion of probes AW-4 and AW-3 (3); after deletion of probes AW-4, AW-3 and AW-15 (4); after deletion of probes AW-4, AW-3, AW-15 and AW-6.

The whole-genome characterization techniques binary typing, PFGE and RAPD analysis display adequate reproducibility among the related genomes of the epidemic MRSA strains (Table 5, clusters V through X). Only the number of repeats within the *spa* gene remain unstable within genetically related strains, and no concordance is demonstrated for analyzing presence versus absence of specific genes (*cna*, *mecA*, *mecI*, and *mecR1*). Strains originated from different locations of hospital F (Table 5, clusters VI and VII) are genetically related, as shown by the genotyping results.

Conclusion and future developments. The binary typing method described herein provides a reproducible, high-resolution molecular typing system strategy that may in the end be preferred over other means of genotyping. This method generates a simply binary output which is to be preferred over the complex banding patterns generated by most other genotyping systems. Furthermore, an important advantage of the binary typing system compared to other genotyping systems is that the system essentially comprises an assay procedure that is amenable to extensive automation and does not require variation in electrophoretic conditions such as voltage, time of run, and temperature, etc. (38, 42). Moreover, DNA hybridization can be performed buy using an enzyme-linked immunosorbent assay-like technique, allowing implementation of this approach in most routine microbiological laboratories. It is theoretically also possible to develop specific DNA probes to determine virulence factors and resistance determinants for additional diagnostic information (15). In principle, this technique can be extrapolated easily to other bacterial species (12). The binary typing system satisfies the requirements of the accepted performance criteria and promises to become a technically simple and fast library typing system.

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Binary typing of *Staphylococcus aureus* strains through reversed hybridization using digoxigenin-ULS® labeled bacterial genomic DNA

Chapter

7

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SUMMARY

Binary typing (BT) of *Staphylococcus aureus* uses DNA probes that react differentially with the genomes of diverse staphylococcal strains. The aim of the present study was to simplify and accelerate the BT procedure. The protocol is based on reversed hybridization of digoxigenin Universal Linkage System (DIG-ULS®) labeled whole genome bacterial DNA extracts to strip-immobilized probe DNA. A high speed DNA extraction procedure for staphylococcal DNA was used. Crude, non-amplified staphylococcal genomic DNA was directly labeled with DIG-ULS. The conditions for hybridization were optimized. After hybridization, a simple chromogenic detection of hybrids was performed. Twenty well-characterized isolates of methicillin-resistant *Staphylococcus aureus* were used as a representative set of tester strains. Five identical, five genotypically related and ten unique *S. aureus* isolates were analyzed. BT confirmed prior genotyping results, based on pulsed-field gel electrophoresis. A technically simple and fast procedure has been developed, paving the way for BT being performed in routine microbiology laboratories.

INTRODUCTION

Over the past years, the development and application of molecular typing systems has initiated a revolution in the characterization of pathogenic microorganisms. Molecular typing has been instrumental in the delineation of clonal expansion of pathogenic or multidrug-resistant bacterial strains, such as for instance *Escherichia coli* O157 (19, 39), methicillin-resistant *Staphylococcus aureus* (33) or multidrug-resistant *Streptococcus pneumoniae* (10, 13). However, these studies were technically complex and the tests used cannot be performed in an average microbiology laboratory. Moreover, most genotyping systems commonly used in microbiology generate complex banding patterns. Consequently, comparison of large numbers of this type of DNA fingerprints and the epidemiological interpretation is tedious and lacks reproducibility (6, 28, 29, 31).

This led to the development of various commercial typing assays, based on nucleic acid probe-reactivity, thereby circumventing the problems surrounding banding pattern standardization and interpretation of conventional fingerprints. The procedures for the use of DNA probes have been technically standardized. Direct probe techniques appear to be of limited utility owing to poor sensitivity. Nucleic acid amplification and signal amplification have been introduced to solve this problem (2, 16, 22, 23, 25). Spoligotyping, a system developed for the genotypic characterization

of *Mycobacterium tuberculosis* and *M. bovis*, encompasses the use of strain differentiating oligonucleotide probes, (4, 16). This technique capitalizes on the detection of polymorphism in the chromosomal direct-repeat (DR) locus. This strategy is of adequate discriminative value (8, 11, 21) and the gain and loss of direct repeats is of unknown epidemiological value. Reliable probe-based microbial typing systems, although several have been developed, are not yet commonplace in microbiological practice. For these and other reasons, we here describe the development of a high-speed, non-radioactive target-labeling format for the binary typing technique as was developed for the characterization of *S. aureus* (36-38). DNA is extracted from overnight *S. aureus* cultures, labeled with digoxigenin Universal Linkage System (DIG-ULS®) (30) and reversibly hybridized to strips containing immobilized strain-differentiating DNA probes. Signal is generated by chromogenic staining and binary types can be read visually. The novel binary typing system for the characterization of *S. aureus* strains will be introduced and its versatility will be discussed.

MATERALS AND METHODS

Bacterial strains. Well-characterized strains of *S. aureus* (n=20) were obtained from a reference collection (31). The collection was composed of 10 genetically unrelated isolates, displaying a unique pulsed-field gel electrophoresis (PFGE) pattern, 5 isolates exhibiting similar but not identical PFGE fingerprints and 5 isolates sharing indistinguishable PFGE patterns. Isolates were cultured onto blood agar plates at least once, and single colonies were used for further testing.

DNA isolation. Firstly, *S. aureus* strains were grown overnight at 37°C on Columbia III agar (Becton Dickinson, Etten Leur, The Netherlands) supplemented with 5% sheep blood. Subsequently, 3 to 5 individual colonies were suspended in 150 µl of 25 mM Tris, 10 mM EDTA, 50 mM glucose (TEG buffer, pH8.0). To prepare spheroplasts, 75 µl lysostaphine solution (100 mg/ml in distilled water, Sigma Chemical Corporation, St. Louis, Mo.) was added and the suspension was incubated at 37°C for 1 h. Finally, staphylococcal DNA was extracted using one out of seven different DNA isolation protocols.

i. **Boom method.** DNA was isolated according to the protocol described by Boom et al (5). Spheroplasts were lysed by addition of 1 ml 4 M guanidinium isothiocyanate, 50 mM Tris-HCl pH 6.4, 3 mM EDTA, 1% Triton-X100. To immobilize and purify the DNA, 50 µl of a Celite suspension (0.2 g/ml, Janssen Pharmaceuticals, Beerse, Belgium) was added. The entire mixture was shaken for 15 sec and incubated at room temperature (RT) for 10 min. After centrifugation, the supernatant was discarded and the pellet was washed. The Celite pellet was dried in vacuo and, subsequently, 100 µl of distilled water was

added. DNA was eluted by incubation at 56°C for 10 min. The supernatant containing the DNA was separated from the Celite by centrifugation.

ii. **Miniprep of bacterial genomic DNA with CTAB/NaCl solution** (3). Spheroplasts were lysed by the addition of SDS and proteinase K (final concentrations; 0.5% and 100 µg/ml, respectively). The suspension was mixed and incubated for 1 h at 37°C. Cell wall debris, denatured proteins and polysaccharides were linked to CTAB as a complex (hexadecyltrimethyl ammonium bromide) / NaCl solution during an incubation step for 10 min at 65°C. The CTAB precipitate was then removed by phenol / chloroform / isoamylalcohol extraction (24:24:1,v/v). The DNA, present in the aqueous phase, was precipitated with 0.6 vol. isopropanol and was pelleted by centrifugation. The pellet was washed with ethanol 70% and respun. Subsequently, the DNA pellet was redissolved in distilled water.

iii. **Extraction with Phenol/Chloroform** (24). The spheroplasts were mixed with an equal volume of phenol / chloroform / isoamylalcohol (24:24:1, v/v) until an emulsion forms and subsequently, the mixture was centrifuged for 15 sec in Eppendorf centrifuge at RT. The aqueous phase was transferred into a new tube and the phenol / chloroform / isoamylalcohol step was repeated. The DNA was precipitated with 0.6 vol. isopropanol and pelleted by centrifugation. The pellet was washed with ethanol 70% and respun. Subsequently, the DNA pellet was redissolved in distilled water.

iv. **DNA isolation through proteinase K / SDS treatment and boiling.** SDS and proteinase (final concentration; 0.5% and 500 µg/ml, respectively) were added to the spheroplasts and incubated for 1 h at 37°C. The lysate was boiled for 10 min.

v. **DNA isolation through boiling.** DNA isolation protocol no. iv was repeated without SDS and proteinase-K treatment.

vi. **Alkaline method for DNA extraction.** Spheroplasts were added to a solution of 0.2N NaOH and incubated for 5 min at RT. The lysate was transferred on a Sephadex G50 column (Pharmacia Biotech Products, Roosendaal, The Netherlands) equilibrated in 10 mM Tris-HCl 0.1 mM EDTA buffer in order to neutralize the suspension. The eluate was collected into a fresh tube.

vii. **Wizard® Genomic DNA purification kit.** The extraction of staphylococcal DNA was performed according to the manufacturer's instructions (Technical manual no. 050, Isolation of genomic DNA from gram positive and gram negative bacteria).

In procedures i to vi, the extracted DNA was divided into two portions prior to precipitation. One part of the preparation was left untreated, whereas the other part was purified by ethanol precipitation. For that purpose, 0.1 vol. 3 M sodium acetate and 2.5 vol. ethanol absolute was added to the DNA solution and the mixture was incubated at -80°C for 1 h. The precipitate was pelleted through centrifugation for 15

min at 4°C. The pellet was carefully washed with ethanol 70% and the centrifugation step was repeated. The DNA pellet was lyophilized and re-dissolved in distilled water.

The DNA size distribution and concentrations of all preparations were estimated by gel electrophoresis through 1% agarose gels in 40 mM Tris-borate, 2 mM EDTA (0.5x TBE), stained with ethidium bromide, and compared to samples containing a known amount of DNA (24). DNA sample portions were diluted to a concentration of 100 ng/µl and 1 µg (10 µl) thereof was used for labeling and hybridization experiments. The remainder was stored at -20°C.

Fragmentation of the DNA. To study the effect of DNA fragment size on labeling efficiency and hybridization dynamics, purified DNA was sonicated three times for 30 sec (amplitude 5; Soniprep 150, Beun de Ronde, Abcoude, The Netherlands) to obtain DNA fragments ranging from 100 to 300 bp. This was confirmed by gel electrophoresis through 1% agarose gels in 40 mM Tris-borate, 2 mM EDTA (0.5x TBE), stained with ethidium bromide.

DNA labeling optimization. Purified *S. aureus* DNA was labeled using the digoxigenin-ULYSIS® nucleotide labeling kit (DIG-ULS®; KREATECH Diagnostics, Amsterdam, The Netherlands). The label was used in three different ratios (µg DNA versus Units ULS), namely 1:1, 1:2, and 1:4. The structure formula of the reagent and labeling reaction is depicted in Figures 1a and 1b. Labeling of target DNA was performed for 30 min at 85°C and any free DIG-ULS® was blocked by adding stop-buffer and incubating for 10 min at RT. A serial dilution of labeled DNA from all strains (1 µl) was spotted onto a membrane; 1000 pg, 300 pg, 100 pg, 30pg, 10 pg, 3 pg, 1 pg, 0.3 pg, 0.1 pg and 0 pg to check the labeling-efficiency. The spotted DNA was conjugated with anti-DIG-alkaline phosphatase, followed by chromogenic detection. In the standard labeling quality control procedure, 1 µg non-labeled fish sperm DNA (average fragment size approx. 200 bp) was labeled with 1U DIG-ULS® following the standard procedure mentioned above.

DNA probes. The procedures for the generation, validation, and application of the 12 strain-specific DNA probes have been described in detail elsewhere (36-38). After amplification of the recombinant plasmids, amplicons were purified according to different methods; ethanol precipitation (24), Quickstep PCR purification (Edge Biosystems, SphaeroQ, Leiden, The Netherlands), or QIAquick™ PCR purification kit (Westburg, Leusden, The Netherlands). The optimal amount of spotted probe DNA was established in a hybridization experiment, using serial dilutions; 150 ng, 100 ng, 50 ng, 25 ng, 10 ng, and 1 ng per spot. The quality of the DNA probe was ascertained by establishment of its molecular size and the specific restriction enzyme pattern.

Table 1. Comparative analysis of characteristics obtained with the binary typing technique and other genotyping methods for the panel of methicillin-resistant *S. aureus* strains (31).

MRSA strain no.	Binary code	PFGE type	TAR916 SHIDA type	AP PCR type by: RAPD with ERIC2	RAPD with AP1	RAPD with AP7	Phagetype
Witte 1	001010011111	A	A	A	A	A	77
Witte 2	001010011111	A	A	A	A	A	77
Witte 3	001010011111	A	A	A	A	A	77
Witte 4	001010011111	A	A	A	A	A	77
Witte 5	001010011111	A	A	A	A	A	77
Witte 6	000110011111	B	B	B	B	B	NT
Witte 7	000010001111	B1	B	B	B	B	95
Witte 8	000010001111	B2	B	B	B	B	80
Witte 9	000010011111	B3	B	B	B	B	80
Witte 10	000110011111	B4	B	B	B	B	NT
Witte 11	001111011111	C	A	C	A	A	75,77,84,84a,994
Witte 12	011111011111	D	C	D	C	C	6,81
Witte 13	001110011111	E	D	E	A	D	6,47,77,83A,85,994
Witte 14	000010011111	F	E	F	D	E	85
Witte 15	001011011111	G	F	E'	C	F	85
Witte 16	011110011111	H	G	G	A	D	6,47,66
Witte 17	101110011111	I	H	H	A	E	75,77
Witte 18	000110011111	J	A	H	A	A	47,54,75,77,84,85,812
Witte 19	000110011111	K	I	I	B	G	29,52,77
Witte 20	110110011111	L	J	F	A	I	29

a Data are from reference 10. Abbreviations: MRSA, methicillin-resistant *S. aureus*; PFGE, pulsed-field gel electrophoresis; TAR916 SHIDA, Transposon 916, Shine Dalgarno; AP PCR, arbitrarily primed PCR; RAPD, randomly amplified polymorphic DNA analysis (ERIC2, AP1, and AP7 are primers); NT, not typeable.

Spotting the DNA probes. All purified DNA probes (n=12) were spotted (1 µL) on a Hybond™ N⁺(Amersham Life Science, Buckinghamshire, UK) membrane strip (5 x 150 mm). The spotted DNA probes were denatured by 0.5 M NaOH / 1.5 M NaCl treatment for 15 min at room temperature. Subsequently, strips were neutralized with 0.5 M Tris-HCl / 1.5 M NaCl, pH 7.4 for 15 min at RT. Strips were washed with 2x SSC (24) and the denatured DNA was crosslinked on the strip with UV light (280 nm).

For hybridization quality control, 10 ng petunia DNA (LMC 1322) and 0.25 ng of *S. aureus* (Witte 2) amplified *nuc* gene (26) was added to the membrane as negative and positive hybridization control, respectively.

Optimization of the DNA hybridization conditions. A serial dilution of purified and randomly labeled staphylococcal DNA (800 ng, 400 ng, 200 ng, 100 ng, and 50 ng) was hybridized with various concentrations of immobilized DNA probes (see DNA probes section).

Hybridization. Each strip was transferred into a Greiner tube (15ml) and 1 ml DIG Easy Hyb buffer (Roche Molecular Biochemicals, Almere, The Netherlands) was added. Strips were pre-hybridized for 1 to 2 h at 42°C in a rotation oven. Labeled DNA was denatured for 5 min at 100°C and quenched on ice. The sample was centrifuged to collect condensate and 1 ml DIG Easy Hyb buffer was added. The probe was then added to the hybridization mixture and was incubated overnight at 42°C in a rotation oven.

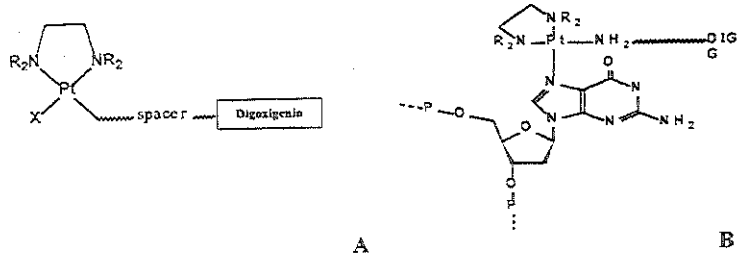

Figure 1. (A) Structural formula of Digoxygenin-ULS®. ULS® consists of a square planar Pt(II)-complex, which is stabilized by a chelating diamine. (B) Labeling reaction between Digoxygenin-ULS® and DNA. Labeling occurs predominantly through binding of the ULS® compound to the N7-position of guanine.

Detection of the hybridized DNA. After hybridization, strips were washed twice in 2x SSC, 0.1% SDS for 5 min at RT and twice in 0.5x SSC, 0.1% SDS for 15 min at 68°C. Strips were blocked with DIG wash and blocking buffer kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. Briefly, after post-hybridization wash steps, strips were equilibrated in 1x maleic buffer for 1 min. Subsequently, the strips were blocked for 30 to 60 min at RT with 1x blocking buffer under slight agitation. Strips were incubated with conjugate (anti-digoxigenin AP conjugate, 150 mU/ml) for 10 min at RT. Strips were washed 2 x 15 min in 1x wash buffer at RT and equilibrated for 2 x 5 min in detection buffer. Finally, strips were incubated in freshly prepared color substrate (NBT/BCIP ready-to-use tablets, Roche Molecular Biochemicals) for maximal 3 h at RT in the dark.

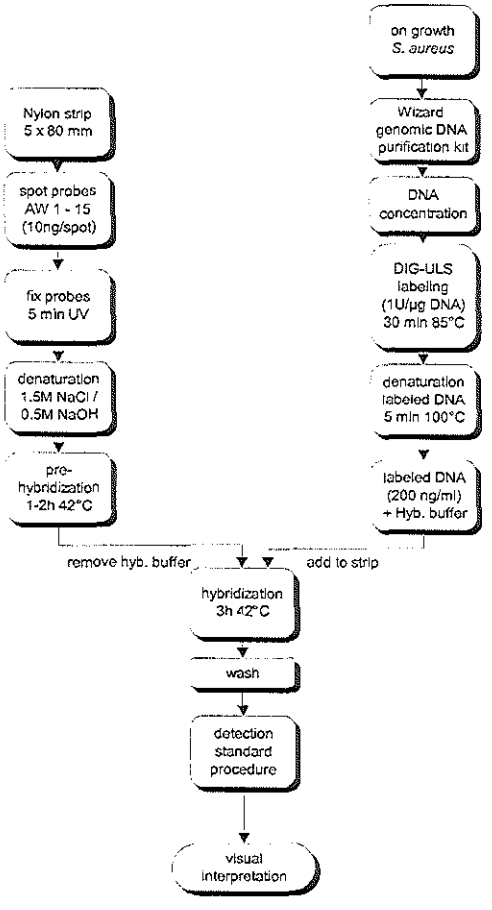

Figure 2. Schematic diagram representing the complete novel binary protocol.

RESULTS

Determination of the optimal reversed hybridization procedure for binary typing.

i. **Labeling efficiency.** Overall, the labeling efficiency increased proportionally with the amount of DIG-ULS® added (data not shown). In a spot blot assay DNA isolation methods no. iii, iv and v displayed a high level of density with a detection limit of 3, 0.3, and 3 pg DNA, respectively. However, small quantities of protein and RNA were still present in these preparations and since these are also labeled with DIG-ULS® they could contribute to false positive

results. Noticeably, the labeling efficiency (method i to vi) decreased dramatically when the ethanol precipitation step was omitted (data not shown). Method vii (Wizard® Genomic DNA purification kit) provided the most convenient purification of genomic DNA for labeling with a detection level of 0.3 pg DNA in a spot blot assay.

ii. **Optimization of the hybridization conditions.**
Hybridization experiments showed that 10 ng probe DNA (purified with QIAquick™ PCR purification kit) per spot in combination with 200 ng labeled genomic DNA (purified with Wizard® Genomic DNA purification kit) per ml hybridization buffer, revealed clear binary hybridization signals. DNA isolation methods iii, v, and vi resulted in significant background and results were hardly interpretable (data not shown). DNA isolation method iv produced adequate quality DNA. However, the hybridization signals remained weak, which was probably due to co-labeling of RNA and protein that were still present in the DNA preparation. The amount of DIG-ULS®, which was added to the DNA, is inverse proportional to the intensity of the hybridization signal and a ratio of template DNA (µg) versus DIG-ULS label (Units) of 1 : 1 yielded the best results.

iii. **Fragmentation of target DNA.** Non-fragmented samples yielded the best hybridization results (data not shown). The most optimal conditions for the binary typing procedure is summarized below and outlined in Figure 1. Genomic staphylococcal DNA was purified with the Wizard® Genomic DNA purification kit and labeled with DIG-ULS® (target DNA versus label in a ratio of 1:1). A concentration of 200 ng labeled DNA per mL hybridization buffer was used. The strain-specific DNA probes were purified with the QIAquick™ PCR purification method and 10 ng (1 µl) of each DNA probe was spotted on a nylon membrane strip. Hybridization quality controls were added to the membrane strip. As a positive control, 0.25 ng (1 µl) amplified *nuc* gene of *S. aureus* Witte 2 (26) and as a negative control, 10 ng (1 µl) petunia DNA (LMC 1322) were applied. Hybridization was performed overnight at 42°C with continuous agitation.

Application of the new binary typing procedure. The hybridization results of the *S. aureus* strain collection, determined with the currently developed reversed hybridization procedure are depicted in Figure 3 and outlined in Table 1. Lanes 1 - 5, referring to the clonal strains, showed an identical binary code. Lanes 6 - 10, representing the genetically related strains, displayed binary patterns that varied in no more than 2 out of the 12 probes. The 10 unique strains (lanes 11 – 20) exhibited unique binary codes, except for strain 18 and 19.

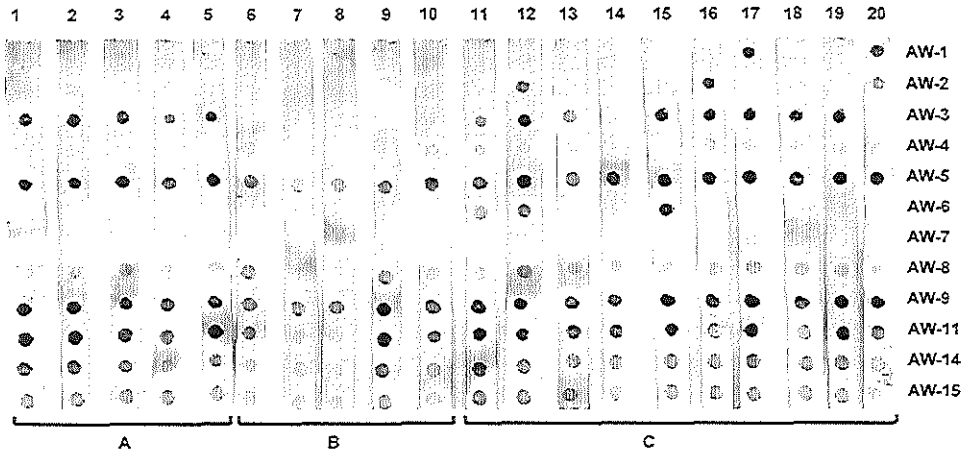

Figure 3. Binary typing results of the MRSA strain collection obtained after hybridization on the strip-immobilized DNA probe panel (AW-1 through AW-15).

DISCUSSION

Several direct microbial detection and characterization techniques using nucleic acid molecules, have been introduced. Line-probe assay is a fast and simple technique and is used for the detection of mutations within resistance genes (17, 23, 40), for the characterization of diverse human pathogens (7, 15), or for the determination of allelic polymorphism among virulence genes (34, 35). Spoligotyping has been developed for the characterization of *Mycobacterium tuberculosis* (16) and is a sensitive technique, due to the multiple repeat units in the DR-target sequence. However, it has an observed moderate discriminatory power (8, 21) probably due to the fact that the system is based on a genomic target with a slow molecular speed (11). PCR in combination with sequence-based techniques, such as multi-locus sequence typing produce detailed results of allelic polymorphism in multiple housekeeping genes (1, 12, 14, 18, 25, 27). At the moment, this technique is complex, expensive and inconvenient for the routine microbiology laboratory. Recently, the RiboPrinter® has been introduced for the characterization of pathogenic microorganism (2). The existing database is not yet adequate (20) and the level of resolution needs to be improved (9).

Here we present a new format of BT as an example of a single species typing test, developed for the characterization of *S. aureus* strains. It goes without saying that the test can be extrapolated to other species. In previous studies, we established the resolution, reproducibility and stability of the epidemiological markers of the binary typing technique (32, 36-38). Technically speaking, the initial method, involving

repeated probe hybridization to digested staphylococcal DNA was complex and time consuming. We therefore developed a simple and fast format for the characterization of *S. aureus* strains based on reversed hybridization with twelve strip-immobilized DNA probes. The major advantage of ULS® labeling for this application is the direct labeling of "culture amplified" total genomic *S. aureus* DNA (target labeling). The additional value of binary typing is its simplicity, speed, reproducibility and no need for expensive peripheral equipment. The efficiency of the labeling reaction with DIG-ULS® (30) depends on the time, temperature, the label-to-DNA ratio and the purity of the DNA. The usefulness of ULS® for labeling of extracted genomic DNA was studied by the application of 7 different DNA isolation protocols. A simple and standardized procedure displayed optimal labeling and hybridization results. We demonstrated that sonication of the genomic DNA did not provide a significant improvement in the labeling efficiency or in the hybridization detection level. Hybridization conditions, probe and target concentration were optimized.

Application of the novel BT protocol. The reformatted binary typing technique was used for the characterization of a *S. aureus* strain collection. All genetically unique *S. aureus* strains displayed a distinct binary code, except for strains 18 and 19. This confirmed the adequate discriminatory power of this method. The stability of the epidemiological determinants was established by the typing results of the epidemiologically linked strains. All produced identical binary patterns. The binary codes of the genetically related *S. aureus* strains were similar, although up to 2 probes show different hybridization results.

Conclusion and future development. The new binary typing protocol described here provides a simple and fast probe-based molecular typing strategy for the characterization of *S. aureus* strains and generates easily interpretable results, which can be compiled in a database. This strategy can be used for the characterization of other species. This "culture-amplified" nucleic acid probe technology can be easily extended with probes that directly detect genes associated with virulence factors and resistance determinants and can be converted to a micro-array based system. It may be concluded that this technique comprises a compact, user friendly *S. aureus* typing system, suitable for the development of an international, communicable database for both MSSA and MRSA. The binary typing system has the clear potential to be useful in peripheral laboratories as well. The inter-laboratory reproducibility of the assay is currently subject of a multi-center study.

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Inter-center reproducibility of binary typing for *Staphylococcus aureus*

Chapter

8

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SUMMARY

The reproducibility of the binary typing (BT) protocol was analyzed in a biphasic multicenter study. In a Dutch multicenter pilot study, ten genetically unique isolates of methicillin-resistant *Staphylococcus aureus* were characterized by the BT assay as presented by van Leeuwen et al. (J. Clin. Microbiol. 2001 39(1):328-331). The BT assay, including the standardized DNA extraction protocol was performed in duplicate in eleven medical microbiology laboratories. Two different detection procedures were applied and a pre-labeled DNA sample as process control were distributed. Only three laboratories accurately identified all strains. Divergence in technical procedures resulted in misinterpretation, due to an increasing number of faint or absent hybridization signals with high background staining. The binary type of the process control was interpreted correctly by all participating laboratories. The feasibility of the BT protocol was related directly to the skill of the laboratory personnel. On the basis of the national study, we concluded that the DNA extraction protocol needed modification to improve DNA yield and purity.

Subsequently, seven European laboratories participated in an international study to determine the reproducibility of the modified BT protocol. Each center was asked to analyze 10 DNA samples previously extracted from 10 MRSA strains (phase 1), and additionally, to analyze 10 MRSA strains, using the standardized or their in-house DNA isolation protocol (phase 2). A pre-labeled DNA process control sample was included for phase 2. The binary types of all DNA samples were identified correctly by all but one laboratories. This latter laboratory diverged from the protocol by adding an excess of labeled DNA to the hybridization mixture, resulting in a high background and therefore non-interpretable BT results. All centers produced identical BT results for the process control. Five of the seven centers correctly identified the binary types of all 10 MRSA strains. Three of these centers used their in-house DNA extraction protocol. Divergence from the standard BT protocol in two centers resulted in no BT data for the 10 MRSA strains. The study demonstrated that each center, that followed the BT protocol correctly, could generate reproducible results irrespective whether or not an in-house DNA isolation protocol was used. The current BT protocol was thus considered to represent a simple method generating robust, reproducible genotype data for *S. aureus* strains.

INTRODUCTION

Over the past two decades, the development and application of molecular typing techniques has initiated a revolution in the characterization of pathogenic and antibiotic resistant *Staphylococcus aureus* strains. A wide variety of image-based approaches including PCR - restriction fragment length polymorphism (PCR-RFLP) analysis of variable regions in specific genes, macro restriction analysis by pulsed-field gel

electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, or amplified fragment length polymorphism (AFLP) was introduced. However, several studies demonstrated that these fingerprint-generating techniques face limitations with respect to reproducibility, standardization, subjective analysis of the images and interpretation of the results (5, 6, 20, 21). These issues make the exchange of strain-typing information between centers difficult and prevent the construction of an international database of *S. aureus* genotypes. A different strategy of strain differentiation is the assessment of the sequence variability in housekeeping genes, the so-called multi-locus sequence typing (MLST) (7, 13) or from genes with a higher evolutionary clock-speed (14, 15). This approach will enable data-exchange between centers. However, these techniques are currently still expensive, technically demanding and therefore restricted to a few reference laboratories. Moreover, the typing results generated with these strategies may not be very helpful to the hospital epidemiologist. In contrast, probe-based methods generating binary results, would appear preferable tools for the analysis of outbreaks or for hospital surveillance systems, monitoring the prevalence of staphylococcal populations over an extended period of time. This strategy could be further enhanced by applying DNA chip technology. Accordingly, we developed a binary typing (BT) method (22-24), based on DNA probes that discriminate between different *S. aureus* genomes. This technique most likely complies with the criteria of a library typing system, i.e. a highly standardized test protocol and output in the form of binary results allowing the assignment of numeric profiles in a reproducible fashion.

The aim of the present study was to determine the feasibility and the reproducibility of our BT system in a multicenter setting. In an initial Dutch pilot study, each of the eleven participating centers performed the entire BT protocol for ten MRSA strains in duplicate. The detection of the hybrids was done with two different substrates. Each laboratory was asked to interpret the binary results obtained with both detection methods and for suggestions and remarks on the test and protocol. The results were collected and co-interpreted by the organizing center. The shortcomings of the BT protocol were addressed and subsequently the improved BT system was tested in an international multicenter study.

MATERIALS AND METHODS

Bacterial strains. Ten MRSA isolates (21, 24) were used in the national multi-center pilot study and in phase 2 of the international multicenter study. This collection was composed of 10 genetically unique strains as assessed by macrorestriction analysis through pulsed-field gel electrophoresis (PFGE). Isolates were stored in the coordinating center (Erasmus MC, MM&ID, Rotterdam, The Netherlands), cultures were encoded and for the national study, stored in Microbank cryovials (Pro-Lab Diagnostics, Lancashire, U.K.) at -20°C. For the international study, cultures were encoded and

separately stored in Trypticase Soy Agar slants (Becton and Dickinson, Etten-Leur, the Netherlands) at 4°C.

DNA samples for the international study. Ten DNA samples, extracted from the genotypically unique MRSA strains (see above), were included to assess the reproducibility of binary typing in phase 1 of the international study. The DNA was extracted from the bacterial strains according to the standardized protocol described in the DNA isolation section (see below). The concentration and purity of the samples were determined by UV spectroscopy by the organizing center and the DNA samples were sent to the participating centers.

DNA isolation protocol for the national multicenter study. Staphylococcal DNA was isolated using a standard protocol (Wizard Genomic DNA purification kit, Promega, Leiden, the Netherlands, (24)). All reagents were distributed to the participating centers. Prior to DNA isolation, several beads from the cryovials, containing the *S. aureus* isolates, were inoculated into Mueller-Hinton broth (Difco, Becton Dickinson Microbiology Systems, Sparks, USA) and after overnight incubation a loopful of broth was subcultured on blood agar plates. Single colonies were reinoculated into fresh Mueller-Hinton broth and incubated for 16-18 h at 37°C under static condition.

The DNA from the grown bacterial cultures were extracted using the Wizard genomic DNA purification kit according to manufacturer's protocol (Promega, Leiden, the Netherlands). Cells were vortexed for resuspension and 1 ml was transferred into a 1.5 ml microcentrifuge tube. Bacterial cells were pelleted, supernatant was removed and the pellet was resuspended into 400 µl 50 mM EDTA. In order to create sphaeroplasts, 200 µl lysostaphine (Sigma, Zwijndrecht, the Netherlands; 100 ng/µl) was added and the suspension was incubated for at least 1h at 37°C, resulting into a clear solution. Subsequently, 600 µl of Nuclei Lysis solution was added, mixed by pipetting and incubated for 5 min at 80°C. The lysate was cooled to room temperature (RT) and RNAse solution (3 µl) was added. The lysate was mixed by inversion and subsequently, incubated for 15 to 60 min at 37°C. Samples were cooled down to RT and 200 µl Protein Precipitation solution added to the RNAse treated lysate. Samples were mixed by vortexing for 20 sec and incubated on ice for 5 min. After centrifugation (3 min at 16.000xg) DNA containing supernatant was transferred into a clean 1.5 ml tube and one volume of isopropanol was added. The tube was mixed by inversion until a visible precipitate was observed, which was pelleted by centrifugation (16.000 xg for 2 min). The DNA pellet was washed with ethanol 70% by gently inverting the sample. The centrifugation step was repeated and the supernatant removed. The pellet was dried under a vacuum for 5 min or air dried (10 to 15 min). Sample rehydration was performed by adding 50 µl rehydration solution followed by incubation at 65°C for 1h.

DNA isolation protocol for the international multicenter study. Several modifications were introduced in the extraction protocol. All reagents of the Wizard genomic DNA purification kit were sent to each center. Prior to DNA isolation, strains were subcultured from the TSA slants (see bacterial strains section) onto agar media

containing 5% sheep blood (Becton and Dickinson). Ten to fifteen colonies were suspended in 400 µl 50 mM EDTA. Moreover, the protein precipitation step including centrifugation was performed twice.

DNA quantification. It was critical to determine the exact concentration of the DNA for efficient Universal Linkage System (ULS)-labeling and optimal hybridization. This was performed by UV spectroscopy using the convention that OD 1 at 260 nm equaled 50 µg/ml of dsDNA. The absorbance was checked at 260 and 280 nm for purpose of DNA concentration and purity analysis and these values did not exceed the range of OD 0.1 to 1.0. As indicator for DNA purification, the ratio (A260/A280) was in the range between 1.7 and 2.0. Samples were diluted in rehydration solution which itself was used as a blank. DNA samples were stored at -20°C until further use.

Binary typing protocol. The procedure for binary typing, as previously described (24) was used with several minor adjustments in hybridization conditions as described below and shown in figure 1. The BT protocol consisted of two steps, namely genomic DNA labeling and filter hybridization with detection.

Labeling of genomic DNA. The DIG-ULS labeling kit (Kreatech Diagnostics, Amsterdam, the Netherlands) was applied for random labeling of *S. aureus* genomic DNA. DIG-ULS (1U) was added to 1 µg staphylococcal DNA and the sample volume was adjusted to 20 µl with labeling solution. Samples were incubated for 30 min at 85°C and cooled subsequently on ice for 1 min. The labeling reaction was stopped with Stop solution (5 µl) after incubation at RT for 10 min. Labeled DNA samples were stored at -20°C until further use.

Filter hybridization and detection. Eleven binary typing strips each containing 12 strain-specific DNA probes, one positive and one negative control were obtained from Kreatech Diagnostics (Amsterdam, the Netherlands). Each strip was coded and incubated with prewarmed hybridization mixture (1 ml) at 42°C in a 15 ml plastic tube. Labeled DNA samples and a process control, containing DIG-ULS labeled staphylococcal DNA, were denatured for 5 min in a 96°C waterbath and cooled subsequently on ice for another 5 min. From each denatured, labeled DNA sample (n=11), 5 µl (200 ng) was added to the hybridization mixture and the tubes incubated for 16-18 h at 42°C in a rolling hybridization oven or in a shaking waterbath. After hybridization, BT strips were transferred into a container with 50-100 ml 0.3M sodium chloride, 0.03M tri-sodium citrate (2x SSC) and 0.1% sodium dodecyl sulphate (SDS) for post-hybridization washing for 2x 5 min at RT on a shaking device. This was followed by two 15 min washing into 50-100 ml 0.5x SSC – 0.1% SDS in a rolling hybridization oven at 60°C. The following blocking and detection steps (Roche Molecular Biochemicals, Almere, the Netherlands) were performed on a shaking device at room temperature (RT). The BT strips were rinsed in 20 ml maleic acid buffer for 1 min, and subsequently, were blocked for 30 min in 1x blocking solution diluted in maleic acid buffer. Hybrids were conjugated with anti-digoxigenin-alkaline phosphatase (150 mU/ml) and strips were incubated for 30 min in 20 ml antibody solution.

Table 1. Comparative quality assessment of the BT data produced by eleven different Dutch laboratories using a preliminary version of the BT protocol.

Cumulative score of DNA probes[a]	Center 1		Center 2		Center 3		Center 4		Center 5		Center 6		Center 7		Center 8		Center 9		Center 10		Center 11	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Samples																						
Correct	89.2	0	41.3	87.9	19.2	16.7	99.2	100	75	24.6	99.2	100	28.3	33.3	15	25	87.5	90	0	0	100	100
Incorrect	0.8	0	3.7	7.1	0.8	3.3	0.8	0	0	0.4	0.8	0	1.7	1.7	0	0	2.5	0	0	0	0	0
Not done	0	100	50	5	0	30	0	0	0	0	0	0	0	0	50	50	0	10	0	100	0	0
No signal	10	0	5	0	80	50	0	0	25	75	0	0	60	65	35	25	10	0	100	0	0	0
Process control[b]																						
Correct	100	0	50	100	100	100	100	100	100	100	100	100	100	100	50	50	100	100	100	0	100	100
Incorrect	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Not done	0	100	50	0	0	0	0	0	0	0	0	0	0	0	50	50	0	0	0	100	0	0
No signal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Detection (preferred method)[c]																						
Chemiluminescent	-		+		+		+		+		-		-		-		-		-			
Chromogenic	+		+		-		+		-		+		+		+		+		+			
DNA extraction protocol[c]	-		+		-		-		-		-		-		-		-		+			
BT protocol[c]	-		+		-		+		+		+		+		+		+		+			
Skill (molecular methods)[d]																						
Student	x		x		x		x															
Technician									x		x		x		x		x		x			
Laboratory	Y		Y		N		Y		Y		Y		Y		Y		Y		Y			

table to be continued on next page

Strips were washed twice in 1x washing buffer (20 ml) for 15 min and subsequently equilibrated in 20 ml detection buffer for 5 min.

BT strips were placed next to each other on a PhotoGene development folder (10 cm x 10 cm; Gibco BRL, Life Technologies, Breda, the Netherlands). CDP-Star detection reagent (approximately 1.5 ml; Roche) was added so that all the BT strips were covered. The development folder was closed and sealed. In a darkroom, an X-ray film was contacted to the folder (DNA side) for a maximum of 1 min. The X-ray film was developed and the results interpreted. The strips were removed from the folder and transferred into a container with 20 ml freshly prepared NBT/BCIP solution (1 tablet per 10 ml distilled water; Roche) and incubated for 3 h in the dark under static conditions. BT strips were washed in 20 ml 10mM Tris-HCl – 3mM EDTA, air-dried on a filter paper, and subsequently, results were visually inspected and interpreted.

Data analysis. BT results, obtained with both detection methods, were interpreted after visual inspection by members of the individual institutes. Binary types were scored as described before (23, 24). Briefly, hybridization of the labeled DNA to the DNA probes were scored with a 1 or a 0 according to the presence or absence of the hybridization signal, respectively. All BT strips, X-ray films and accompanying interpretations were sent to the organizing center for interlaboratory comparison. To evaluate center-to-center reproducibility, the score for each probe for each strain was compared with the BT results of the reference center.

In a questionnaire survey, each institute was asked for comments on the feasibility of the standardized DNA isolation procedure and the binary typing protocol, on their experience with the detection methods, DNA isolation techniques, UV spectroscopy or Southern hybridization and on personnel skill with molecular microbiology techniques.

RESULTS

Analysis of the national multicenter study. Table 1 summarizes the visual interpretations of the BT analysis, as obtained with chromogenic and chemiluminescent detection. The interpretation of the results by each center was verified by

Note. Each center performed the complete test in duplicate, indicated as A and B.
[a]) The probe score in percent is composed of the binary typing results from 10 MRSA strains, as performed by each center. Every MRSA strain is characterized by 12 DNA probes (23) and the hybrids are detected by two different hybridization substrates. A complete consensus of each test with the results from the reference laboratory values 240 (10 strains, 12 probes and 2 detection methods) as a 100% correct score. Incorrect is the percent aberrant probe score; no signal indicates the percent not interpretable hybridization signal.
[b]) Process control are scores using labeled staphylococcal DNA, prepared by the organizing center and distributed among the participating centers.
[c]) +, feasible; -, not feasible; outcome of the questionnaire with respect to the feasibility of the diverse protocols; preferred detection technique, DNA extraction protocol and BT labeling and hybridization protocol.
[d]) Selection of performer's skill, student or technician. Yes (Y) or no (N) experience of the participating center with molecular biology techniques.

central analysis. Three centers (centers 4, 6 and 11) correctly identified the 10 MRSA strains with both detection techniques. The hybridization signals were clear without any observed background. The BT results of the seven other centers (centers 1 - 3, 5, 7 – 9) were more difficult to interpret, by reason of a faint or lacking signal, overexposure of the film after chemiluminescent detection or because of a high background staining. The DNA yield obtained with the standard DNA extraction protocol performed by these seven laboratories was low. Two of these centers (centers 5 and 8) used their own, non-standardized, in-house DNA isolation protocols, which yielded highly impure of the DNA samples. This DNA cold not efficiently labeled. One center (center 10) estimated the DNA concentration by interpolation based on agarose gel electrophoresis of a reference series of lambda-DNA. This method is not sufficiently accurate to allow reliable DNA labeling. Centers 1 and 10 did not perform the second test through lack of sufficient time. The BT chemiluminescent detection was not completed by two centers because of non availability of equipment (center 2, overexposed films; center 8, no developing machine available). Noticeably, all centers correctly identified the binary type of the process control DNA, supplied by the organizing laboratory. The outcome of the questionnaire survey is outlined in the lower part of Table 1. All laboratories, except for center 3, were familiar with molecular microbiology methods, although four centers (centers 2, 5, 6 and 10) only had experience with routine (commercially supplied) diagnostic DNA amplification techniques. In four centers (centers 1 – 4) the test was performed by a student-technician. Four centers (centers 2 – 5) preferred the chemiluminescent detection method by reason of higher sensitivity, while the other laboratories favored the chromogenic detection approach for its simplicity and logistic reasons.

Analysis of the international multicenter study. Table 2 summarizes the BT results as interpreted by each center. Separately, these data were concordant with those of the interpretation by the organizing center. The study was divided into two phases. In the first phase, the BT profile of the staphylococcal DNA samples (n=10) included in the testkit were correctly identified using both detection methods by each center. One center (center 5) diverged from the protocol by the addition of an excess of labeled DNA to the hybridization buffer. This resulted in a high background of the binary patterns obtained with both the chromogenic and the chemiluminescent detection methods. For this reason, the hybridization patterns were not interpretable. In phase 2 of the study, all 10 MRSA strains were correctly classified with the modified BT protocol in 5 out of the 7 European centers. Three of these five laboratories (centers 3, 4 and 6) used their own standardized in-house protocol for the extraction of staphylococcal DNA. The positive hybridization signals of probe AW-2 for MRSA strain 2 and probe AW-4 for the same strain were not recognized by center 3 and 4, respectively. Center 2 failed to obtain clear interpretable results, due to the addition of an excess labeled staphylococcal DNA to the hybridization mixture. For lack of time, center 5 was not able to complete phase 2 of the study. The binary type of the process control as obtained by each center were accurately determined.

Table 2. Comparative quality assessment of the BT data produced by seven European centers using a modified version of the BT protocol.

Cumulative score of DNA probes[a]	Center 1		Center 2		Center 3[c]		Center 4[c]		Center 5		Center 6[c]		Center 7	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
Samples														
Correct	100[*3]	100	100	0	100	99.6	100	99.6	0	0	100	100	100	100
Incorrect	0	0	0	0	0	0.4[*1]	0	0.4[*1]	0	0	0	0	0	0
Not interpretable	0	0	0	100[*2]	0	0	0	0	100[*2]	0	0	0	0	0
Not done	0	0	0	0	0	0	0	0	0	100	0	0	0	0
No signal	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Process control[b]														
Correct		50		100		100		100		100		100		100
Incorrect		0		0		0		0		0		0		0
Not done		50[d]		0		0		0		0		0		0
No signal		0		0		0		0		0		0		0

Note

a) The probe score in percent represents the binary typing results from 10 DNA samples obtained in phase 1 of the study and acquired from the 10 MRSA strains from phase 2, as performed by each center. Every DNA sample and MRSA strain is characterized by 12 DNA probes (23) and the hybrids are established with two different detection methods. Fully concordance of each test with the results from the reference laboratory yield a CORRECT score of 100% (10 DNA samples or strains, 12 probes and 2 detection methods values 240). Incorrect, percentage discrepant probe score; no signal, not interpretable hybridization signal. (1) one discrepant results with probe AW-4 as detected with chemiluminescent substrate in phase 2, chromogenic detection scored 100%; (2) not interpretable signals in phase 1, too much DNA was included and resulted in high background; (3) results after repeated test, first experiment resulted in 80% correct and 20% no signal due to hybridization conditional problems.

b) Process control; labeled staphylococcal DNA as prepared by the organizing center and distributed among the participating centers.

c) Centers 3, 4 and 6 used their in-house standardized DNA extraction protocol.

d) Chromogenic detection of the process control was not performed.

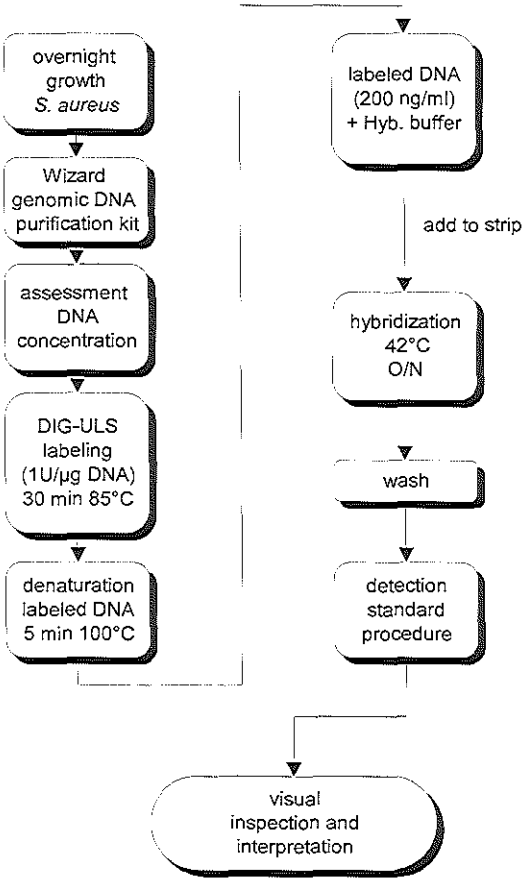

Figure 1. Schematic flow diagram of the complete BT protocol.

DISCUSSION

Accurate epidemiological typing is of primary importance for the identification and tracking the dissemination of *S. aureus* particularly those resistant to methicillin (MRSA) (1-4, 8, 16-19). Currently, a wide array of molecular techniques is available for differentiating *S. aureus* at the strain level. However, these fingerprint generating systems remain difficult to standardize between different laboratories (5, 6, 20, 21). The limitations in reproducibility of an image-based typing technique has for instance clearly been demonstrated in a multicenter study, organized by the European working group of *Legionella* infections (EWGLI). A highly standardized AFLP protocol was tested and the results from the EWGLI members showed considerable variation and discrepancy in

type designation (9, 10). Typing systems that produce a binary output are expected to replace the image-based techniques in the near-future (19). A reason for this is the simplicity of data management (data storage, - exchange, etc.) and the possibility to highly standardize such methods. An example of such a standardized typing technique is spoligotyping for the characterization of *Mycobacterium tuberculosis* isolates (11, 12).

We recently developed a technique for the characterization of *S. aureus* strains, based on strain-differentiating DNA probes, generating a binary output (23, 24). When applied in ten Dutch centers, participating in the pilot study, most of these laboratories were unable to produce clear and interpretable results. Mainly caused by the use of the standardized Wizard DNA extraction protocol, supplied by the organizing center and of diverse non-standardized in-house DNA extraction procedures. The majority of the participating centers reported a very low DNA yield. For this reason, two centers used their own DNA isolation protocol but their efforts resulted in impure DNA preparation. Since DIG-ULS will not only link to ss- and dsDNA, RNA and proteins can also be labeled, impure DNA samples will lead to non-specific labeling, resulting in poorly interpretable results. Two centers determined the DNA concentration of the samples on agarose gels. This resulted in rather inaccurate measurement of the DNA concentration, DNA/DIG-ULS ratio and consequently, in poor DNA labeling efficiency. Other reasons for non-interpretable BT results were over-exposure of the X-ray film after detection of the hybrids using the chemiluminescent substrate and a high background that was noticed after detection with both chromogenic and chemiluminescent substrates, due to addition of an excess of labeled DNA to the hybridization mixture. Following the BT protocol strictly, three Dutch centers were able to determine the binary types of the MRSA strains completely consistent with the results of the organizing laboratory. In these cases, chromogenic detection of the hybrids showed the best results. However, the use of chemiluminescent substrate revealed a higher sensitivity. It must be emphasized that all centers were able to obtain reproducible and well-interpretable results when they used the control DNA samples provided. We are therefore tempted to conclude that technically the reproducibility of the BT labeling, hybridization and detection protocol is good. If the outcome of the typing results was compared with the answers from the questionnaire, it is obvious that BT does need appropriate technical skill and laboratory facilities for molecular biological techniques. However, even a limited experience with BT may improve the results as was noticed in the second test. The majority of the laboratories shared the opinion that the DNA extraction protocol was fairly laborious and consisted of too many steps. In contrast, most centers judged the BT protocol to be a simple and feasible technique.

The basis of every laboratory method is reproducibility. Different laboratories are expected to generate the same results and exchange the data after performing a test. Based on this criterion, the present study demonstrates that standardization of the BT procedure can be achieved, on the condition that the DNA extraction protocol is strictly followed and pure DNA preparation of known concentration one used in the assay.

Only in this way genomic DNA labeling will be efficient and result in a clear hybridization signal.

The pitfalls with the standardized DNA extraction protocol noted in the pilot study were corrected and the BT protocol was re-evaluated in an international multi-center study. In phase one of this study, six out of the seven centers were able to identify the BT pattern of the DNA samples from the MRSA strains, provided by the organizing center, correctly. Not accurately following the labeling protocol, one center failed to obtain interpretable results. Introduction of the modified DNA extraction procedure in the second phase of the international study led to insufficient DNA yields in some of the participating laboratories. Potential causes for this low yield may be the poor dehydration of extracted DNA owing to co-isolation of polysaccharides or because of the use of temperature sensitive enzymes (personal communication, Promega technical service). For that reason, three centers used their in-house microcolumn-based DNA extraction methods, resulting in excellent BT scores. BT, thus, needs a high level of standardization in preparing the sample DNA. Therefore, some experience with the technique is necessary. There is no clear preference in the choice of detection strategy. Chemiluminescent detection is preferred for reasons of sensitivity or the possibility of adjusting the optimal signal and background ratio. The advantages of chromogenic detection of the hybrids are its simplicity, ease-of-use and interpretation.
A recent PFGE multi-center study (21) stated that the diverse PFGE data sets generated in different centers did not lend themselves to numerical analysis. Work is currently underway to address this, but the technique is inherently complex. Other multicenter studies have shown that the variability of arbitrarily primed PCR (AP-PCR) produced highly discrepant results and data between laboratories could not be compared (6, 20). The high level of concordance in the BT data between the participating centers observed in the international study, hold much promise for BT typing and a method that can yield exchangeable typing data.

In conclusion, this study has demonstrated that each center can produce reproducible BT data, provided that the protocol is strictly adhered to and that a highly standardized DNA isolation technique is used. The BT method described here, is considered to be simple and reproducible.

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General discussion

Chapter 9

In essence, strain typing determines the genetic diversity within the bacterial species. The genetic diversity reflects the accumulation of random mutations resulting from diverse mechanisms, including single base pair substitutions, chromosomal rearrangements, the insertion or deletion of genetic elements, and the horizontal transfer of DNA from the same and even different species. Current molecular techniques can detect subtle alterations or even single genetic events within the bacterial genome. Nevertheless, the analysis of clinically relevant microorganisms, such as *Staphylococcus aureus*, remains a substantial challenge. Because of the selective pressure on specific microbial genetic elements, such as virulence factors and antimicrobial resistant determinants, the human pathogens within a species may represent only a subset of the genotypes within the species as a whole, including commensal, animal, and environmental strains. The utility and effectiveness of strain typing methods are also influenced by the population genetics of the distinct species being studied. *S. aureus* strains are typically considered to have a clonal population structure with many diverging genetic lineages. The species could be represented as an evolutionary tree with discrete branches. MRSA represent a *S. aureus* subset of unique epidemiologic importance and has been studied in detail. Molecular analysis has provided insight in the nature of the resistance genes and their mode of dissemination. These studies demonstrate the value of molecular procedures for the identification and tracking multiresistant *S. aureus* strains. A clear advantage of molecular typing of *S. aureus* strains over the well-established phenotypic typing procedures, is that genetic markers are more stable, can be detected in a reproducible manner, and are not affected by environmental pressure. Many different genetic typing techniques evolved during the last decades, all with their specific profits and drawbacks. Aspects of performance and convenience determine the value of a typing system for general application in use. Current approaches are expensive and complex. Simplicity, speed and being available for routine microbiology laboratory are prerequisites for improvements in the efficacy of typing procedures. Moreover, contemporary techniques yield hardly interpretable, complex fingerprints and probe-based typing strategies should be developed to circumvent these problems.

The use of PCR for amplification of ribosomal sequences and deduction of specific ribosomal sequences provides an excellent sample of how DNA-based diagnostics can be defined to define species nature. Theoretically, similar probe-based techniques can also be used to distinguish among strains within a species. In **chapter 2** we describe the preliminary phase in the development of a new typing system for *S. aureus* based on a simple binary output from DNA probes that display strain-specificity. This PCR-mediated approach is based upon the development of DNA probes that are selected by RAPD genome scanning. Via RAPD analysis , we tried to identify DNA regions in the *S. aureus* genome that enable the isolation of strain-specific DNA probes. This type of discriminatory DNA probes hybridize in a plus/minus fashion with complete genomic DNA molecules from different staphylococcal strains. RAPD fragments that were amplified in only a subset of *S. aureus* DNA preparations were selected. Hybridization studies subsequently revealed that only approximately 12% of these fragments displayed

the desired molecular typing characteristics. This implies that appearance of most of the other RAPD fragments were the result of annealing site mutations or minor insertional and/or deletional events. The nature of the differentially amplified DNA in *S. aureus* was determined and only one probe represent DNA fragments previously known to be related to mobile genetic elements. This is the transposase encoded by IS*257*. The other probes were of previously unknown origin. The usefulness of the current panel of the strain-specific RAPD-derived DNA probes was evaluated by using a well-characterized collection of epidemiologically and genetically (un)related *S. aureus* strains. Both MRSA and methicillin-sensitive *S. aureus* are typeable by all probes. It was demonstrated that by summarizing hybridization events into a binary typing code, a moderate agreement with the epidemiological and typing data was obtained. The identity values of a single probe among the strains, originating from an outbreak, are highly encouraging. The overall resolution among the unrelated strains, however, was decreased. To improve the reproducibility and discriminatory power of our typing method, the study is focused on expanding the number of probes and simplify the hybridization-based test format.

In **chapter 3** the preliminary set of DNA probes, representing the binary typing format and other genotyping techniques were used for the analysis of genetic diversification in relation with prolonged geographical dissemination of *S. aureus* strains. During an outbreak of *S. aureus* in a hospital ward, strains undergo short-term dissemination and the accumulated but nonetheless limited genetic variation can be measured effectively by binary typing. Nationwide dissemination of MRSA (1992-1994) occurred over a longer period. For this reason, an increasing number of polymorphisms could be detected, even with procedures such as pulsed-field gel electrophoresis (PFGE). Whole genome typing procedures detect increasing genetic variation as a result of pronounced dissemination of an MRSA clone. Short-term dissemination does not seem to affect specific loci such as *mecA* and *mec* regulator genes. The worldwide collection of MRSA strains display polyclonality as a consequence of long-term dissemination and horizontal gene transfer. Moreover, due to the continuous spread, distinct entities such as *mecA* and Tn*554* displayed the highest genetic heterogeneity detected in our study. This study shows that, for specific purposes such as epidemiological surveillance or outbreak investigation, the choice of the molecular typing procedure should take into account the level of geographic dissemination.

In **chapter 4** the preliminary binary typing system was applied to trace the dissemination of a clone of MRSA among Turkish hospitals. We have shown that a major MRSA clone circulates in hospitals in the Ankara and Bursa regions of Turkey. With the help of PFGE and binary typing, the genetic homogeneity among Turkish MRSA strains is well established. Apparently, the MRSA strain involved is very well adapted to the clinical setting and is capable of dissemination and local maintenance. When analyzing the strains from Bursa and comparing them to the ones from Ankara, subtle genetic differences were observed: strains from Bursa tend to cluster separately, but are not distant. Again, increasing genetic variation was detected as a consequence of dissemination.

In **chapter 5**, PFGE patterns and binary codes for 38 isolates of *S. aureus* from bovine mammary secretions were determined and compared. The results of both typing techniques were concordant. BT data were easier to interpret then the complex banding patterns generated by PFGE. Moreover, the computer-aided interpretation of the multiple gels is tedious and lack reproducibility. Only a limited number of predominant *S. aureus* clones were found among the diverse herds, emphasizing the results of other studies. The genotypes of bovine *S. aureus* strains were compared to those of human origin. The majority of the strains were host-specific and this imply that successful transfer of bacterial clones between human and cattle is not very frequent.

The aim of the study, described in **chapter 6** was to validate the use of strain-differentiating DNA probes for the genotyping of *S. aureus*. The number of DNA probes has been extended to a number of 15 and have shown the typing system to have a very high index of reproducibility, stability (100%), and discriminatory power (D = 0.998). Hybridization studies revealed that only 12% of the RAPD amplicons, visually selected for uniqueness, exhibited the desired genetic typing characteristics for *S. aureus* strains. Primer site variation may be the origin of the remaining 88% of the differentiating amplicons. The nature of the DNA probes remains largely unknown. The investigation of further alignments awaits publication of the whole *S. aureus* genome sequence. The stability of the DNA probes was evaluated with sequential isolates recovered from healthy individuals who were shown to be persistent nasal carriers of *S. aureus*. The persistent carriers were monitored in 1988 and 1995, and similarities of thee genotypes among these two periods were determined with binary typing, PFGE, and RAPD analysis. The 15 epidemiological markers indicated a high degree of genomic stability over the years, as it was confirmed by the other genotyping techniques. During laboratory storage and replication, mutations and transpositional recombination may occur and the stability of the epidemiological markers for the staphylococcal genome can be measured by in vitro stability. This was estimated by comparing the genomes of strains before and after 50 serial passages of strains on culture media. All DNA probes generated identical results after repeated testing. The Simpson index of diversity (D) expresses the discriminatory power of a genotyping system. Hunter proposed that the standardized discrimination index determines the discrimination index of a typing system that had a reproducibility of 95%; this is designated D_{95}. Binary typing exceeds the level of D_{95}, and consequently this method can be used as a single method. Less discriminating systems can be used in a combination to obtain a significant D_{95} index. The probability of clonal linkage among epidemic strains determined to be similar by diverse genotyping techniques can be expressed at the level of reproducibility. In fact an application of in vivo stability, i.e., comparison of sequential isolates, recovered along the course of an outbreak. The whole genome characterization techniques such as binary typing, PFGE and RAPD analysis display adequate reproducibility among the related genomes of the epidemic MRSA strains.

The initial method, involving repeated probe hybridization to digested staphylococcal DNA, was technically complex and time consuming. The development of

a simple and fast format for the characterization of *S. aureus* strains is described in **chapter 7**. The novel technique is based on reversed hybridization with twelve strip-immobilized DNA probes. The major advantage of Universal Linkage System (ULS®) labeling for this application is the direct labeling of "culture amplified" total genomic *S. aureus* DNA. The additional value of binary typing is its simplicity, speed, and reproducibility. The efficiency of the labeling reaction with DIG-ULS® depends on the time, temperature, the label-to-DNA ratio and the purity of the DNA. A simple and standardized DNA extraction procedure resulted in optimal labeling and hybridization results. Hybridization conditions, probe and target concentration were optimized. The reformatted protocol was tested on a *S. aureus* collection. The data obtained with the novel protocol are identical to those that were obtained with the conventional binary typing procedure. Epidemiologically linked strains were again identified as clusters, whereas unique strains were well-differentiated. The reversed hybridization data are corroborated by those obtained with PFGE and RAPD analysis.

The intercenter reproducibility of the current BT protocol, as analyzed in a biphasic multicenter study, is outlined in **chapter 8**. When initially applied in eleven Dutch centers, most of these laboratories were unable to produce clear and interpretable results, mainly caused by the use of the standardized Wizard DNA extraction protocol, supplied by the organizing center. The majority of the participating centers reported a very low DNA yield. For this reason, two centers used their own DNA isolation protocol but their efforts resulted in impure DNA preparation. Since DIG-ULS will not only link to ss- and dsDNA, RNA and proteins can also be labeled. Therefore, impure DNA samples will lead to non-specific labeling and results in a poorly interpretable signal. Determination of the DNA concentration of the samples on agarose gels, resulted in rather inaccurate measurement of the DNA concentration, DNA/DIG-ULS ratio determination and, consequently, in poor DNA labeling efficiency. Other reasons for non-interpretable BT results were over-exposure of the X-ray film after detection of the hybrids using the chemiluminescent substrate and a high background that was noticed after detection with both chromogenic and chemiluminescent substrates, due to addition of an excess of labeled DNA to the hybridization mixture. Following the BT protocol strictly, three Dutch centers were able to determine the binary types of the MRSA strains completely consistent with the results of the organizing laboratory. In these cases, chromogenic detection of the hybrids showed the best results. However, the use of chemiluminescent substrate revealed a higher sensitivity. It must be emphasized that all centers were able to obtain reproducible and well-interpretable results when they used the control DNA samples provided. We are therefore tempted to conclude that technically the reproducibility of the BT labeling, hybridization and detection protocol is good. If the outcome of the typing results was compared with the answers from the questionnaire, it is obvious that BT does need appropriate technical skill and laboratory facilities for molecular biological techniques. However, even limited experience with BT may improve the results as was noticed in the second test. The majority of the laboratories shared the opinion that the DNA extraction protocol was fairly laborious

and consisted of too many steps. In contrast, most centers judged the BT protocol to be a simple and feasible technique.

The basis of every laboratory method is reproducibility. Different laboratories are expected to generate the same results and, enabling them to exchange the data after performing a test. Based on this criterion, the present study demonstrates that standardization of the BT procedure can be achieved, on the condition that the DNA extraction protocol is strictly followed and pure DNA preparation of known concentration one used in the assay. Only in this way genomic DNA labeling will be efficient and result in a clear hybridization signal.

The pitfalls with the standardized DNA extraction protocol noted in the pilot study were corrected and the BT protocol was re-evaluated in an international multi-center study. In phase one of this study, six out of the seven centers were able to identify the BT pattern of the DNA samples from the MRSA strains, provided by the organizing center, correctly. Not accurately following the labeling protocol, one center failed to obtain interpretable results. Introduction of the modified DNA extraction procedure in the second phase of the international study led to insufficient DNA yields in some of the participating laboratories. Potential causes for this low yield may be the poor dehydration of extracted DNA owing to co-isolation of polysaccharides or because of the use of temperature sensitive enzymes. For that reason, three centers used their in-house microcolumn-based DNA extraction methods, resulting in excellent BT scores. BT, thus, needs a high level of standardization in preparing the sample DNA. Therefore, some experience with the technique is necessary. There is no clear preference in the choice of detection strategy. Chemiluminescent detection is preferred for reasons of sensitivity or the possibility of adjusting the optimal signal and background ratio. The advantages of chromogenic detection of the hybrids are its simplicity, ease-of-use and interpretation.

The high level of concordance in the BT data between the participating centers observed in the international study, holds promise for BT typing as a method that can yield exchangeable typing data.

In conclusion, this study has demonstrated that each center can produce reproducible BT data, provided that the protocol is strictly adhered to and that a highly standardized DNA isolation technique is used. The BT method described here, is considered to be simple and reproducible.

Final conclusion and future perspectives. The binary typing method described in this thesis satisfies the requirements of the accepted performance and convenience criteria. The binary output is amenable for extensive automation (data storage and exchange of data). It is possible to add DNA probes to determine virulence factors and resistance determinants for additional diagnostic information. In principle, this technique can be extrapolated easily to other bacterial species.

SUMMARY

Since the last decades, an array of technologies suited for the characterization of *Staphylococcus aureus* at the strain level has become available. These technologies provide data that can be used for the elucidation of dissemination routes of individual *S. aureus* lineages. However, there is still no consensus as to the optimal procedure for typing staphylococci. Most of the assays tested lack inter-center reproducibility and generate complex banding patterns which are difficult to reproduce and to interpret. This indicates that there is no typing system for the establishment of networks for (inter)national epidemiological surveys and it is foreseen that all procedures that generates fingerprints will in the end be displaced by procedures that produce a binary output.

This thesis describes the development, validation and application of a molecular typing system that generates a binary output, the binary typing (BT) system. The development of DNA probes suitable for differentiating *S. aureus* strains is presented in **chapter 2**. Therefore, randomly amplified polymorphic DNA (RAPD) patterns were analyzed for a collection of *S. aureus* strains. All fingerprints were examined for unique amplicons, and the nature of these DNA fragments was investigated. DNA sequences were determined and homologies were discovered with various known *S. aureus* sequences. Most of the fragments were of previously unknown origin and a few fragments demonstrate the capacity to differentiate strains on the basis of the presence or absence of the sequence element in the staphylococcal genomes involved. Latter remained that, in a 5-digit typing system, accurately distinguished epidemiologically related and unrelated strains of *S. aureus*.

The extent of genome evolution among methicillin-resistant *S. aureus* (MRSA) strains were examined in **chapter 3**. A collection of strains, comprising locally, nationally and worldwide disseminated lineages, was analyzed. Genotypes were determined by whole genome typing techniques and procedures which analyze polymorphism in specific DNA fragments only. The outbreak strains showed subclonal variation, whereas the nationally spreading MRSA strains displayed an increased number of genotypes. Among the worldwide disseminated MRSA strains, the different genotyping techniques revealed a wide heterogeneity of types. Different typing techniques appeared to show different levels of resolution among the three collections of MRSA strains, which could be correlated with the extent of geographical spread. Binary typing and randomly amplification of polymorphic DNA analysis appears to be the typing methods of choice for determining (non) identity among strains that have a recent common ancestor and have undergone yet limited dissemination.

The nationwide spread of a clone of MRSA among Turkish hospitals was described in **chapter 4**. A collection of 39 MRSA strains derived from six different hospitals in Ankara and a single hospital in Bursa, Turkey, were analyzed by multiple genotyping techniques. In agreement with the other genotyping assays, binary typing

identified genetic homogeneity among all MRSA strains studied. It is concluded that a major clone of MRSA has spread through a large part of Turkey.

The application of the BT system for epidemiological study of *S. aureus* strains of bovine origin is outlined in **chapter 5**. Thirty-eight bovine mammary *Staphylococcus aureus* isolates were genotyped by pulsed-field gel electrophoresis (PFGE) and with BT. Both techniques documented the genetic relatedness of the strains in a concordant way. Based on practical and epidemiological considerations, binary typing was preferred. The binary types of bovine isolates strains were compared to previously characterized genetically unique human *S. aureus* strains. Distinct genetic clusters were predominantly associated with a single host species, but some lineages contained strains of both human and bovine origin. PFGE and binary typing can be successfully applied for genetic analysis of *S. aureus* isolates from bovine mammary secretions, for the elucidation of population structure of bacteria within and between host species, and for identification of sources and transmission routes of bovine *S. aureus*.

In **chapter 6** the usefulness of all strain-specific DNA probes, circumventing the BT technique, is presented and validated according to the performance criteria for molecular typing systems. *S. aureus* strains (69% MRSA) were used for the validation of the binary typing system. The 15 DNA probes provided stable epidemiological markers. The discriminatory power of binary typing exceeded that of pulsed-field gel electrophoresis and RAPD analysis. Reproducibility, measured by analyzing multiple strains belonging to a multitude of different epidemological clusters, was comparable to that of other genotyping assays used.

A novel binary typing protocol, based on reversed hybridization of digoxigenin-ULS® labeled bacterial DNA to 12 strip-immobilized probes, is presented in **chapter 7**. The aim of this study was to simplify and accelerate the binary typing procedure. A high speed DNA extraction procedure for staphylococcal DNA was used. Crude, non-amplified staphylococcal genomic DNA was directly labeled with DIG-ULS®. The conditions for hybridization were optimized, and a simple chromogenic detection of hybrids was performed. Five identical, five genotypically related and ten unique *S. aureus* strains were used as a representative set of tester strains. Binary typing confirmed prior genotyping results in a relative fashion.

The reproducibility of the binary typing (BT) protocol was analyzed in a biphasic multicenter study (**Chapter 8**). In a Dutch multicenter pilot study, ten genetically unique isolates of methicillin-resistant *Staphylococcus aureus* were characterized by the BT assay and this was performed in duplicate in eleven medical microbiology laboratories. A pre-labeled DNA sample as process control was distributed. Only three laboratories accurately identified all strains. Divergence in technical procedures, mainly in the DNA extraction procedure, resulted in misinterpretation. The BT of the process control was interpreted correctly by all participating laboratories. The feasibility of the BT protocol was related directly to the skill of the laboratory personnel. On the basis of the national study, we concluded that the DNA extraction protocol needed modification to improve DNA yield and purity.

Subsequently, seven European laboratories participated in an international study to determine the reproducibility of the modified BT protocol. Each center was asked to analyze 10 DNA samples previously extracted from 10 MRSA strains (phase 1), and additionally, to analyze 10 MRSA strains, using the standardized or their in-house DNA isolation protocol (phase 2). A pre-labeled DNA process control sample was included for phase 2. The binary types of all DNA samples were identified correctly by all but one laboratories. This latter laboratory diverged from the protocol. All centers produced identical BT results for the process control. Five of the seven centers correctly identified the binary types of all 10 MRSA strains. Three of these centers used their in-house DNA extraction protocol. Divergence from the standard BT protocol in two centers resulted in no BT data for the 10 MRSA strains. The study demonstrated that each center, that followed the BT protocol correctly, could generate reproducible results irrespective whether or not an in-house DNA isolation protocol was used.

Concluding remarks. The preliminary BT protocol, existing of 5 DNA probes, was successfully used for the characterization of (un)related *S. aureus* strains. After extension of the number of DNA probes, BT proved a highly discriminating, stable and reproducible method for typing *S. aureus* strains under the species level. Subsequently, the BT procedure was developed in an efficient and reproducible technique, which was affirmed in a multicenter study.

SAMENVATTING

Gedurende de afgelopen decennia is er voor het karakteriseren van *Staphylococcus aureus* op stam niveau een groot aantal technieken ontwikkeld. Deze technieken leveren gegevens op, welke gebruikt kunnen worden om meer inzicht te krijgen in de verspreiding van afzonderlijke *S. aureus* stammen. Er is echter geen eenduidig oordeel over de ideale methode voor het typeren van stafylokokken. De meest gebruikte methoden hebben een beperkte interlaboratorium reproduceerbaarheid en genereren bovendien complexe bandenpatronen, welke lastig zijn te interpreteren. Dit houdt o. a. in, dat er momenteel geen typering systeem voorhanden is voor de ontwikkeling van (inter) nationale netwerken voor het uitwisselen van typeringsresultaten voor epidemiologisch onderzoek. Het is om die reden te verwachten, dat systemen welke bandenpatronen produceren zullen worden vervangen door technieken, die binaire gegevens genereren. Wij ontwikkelden een typering systeem, dat gebaseerd is op DNA probes welke een differentiële reactiviteit voor *S. aureus* genomen vertonen. De positief/negatief (1/0) resultaten vormen een binair getal en is het resultaat van wat het zgn. binair typering systeem (BT) genoemd.

In dit proefschrift wordt de ontwikkeling, validatie en toepassing van een moleculair typering systeem voor *S. aureus* beschreven. De ontwikkeling van de individuele DNA probes, welke geschikt zijn voor het BT van *S. aureus* stammen, wordt beschreven in **hoofdstuk 2**. Hiervoor werden *S. aureus* stammen geanalyseerd m.b.v. de randomly amplified polymorphic DNA (RAPD) methode. Alle bandenpatronen werden onderzocht op unieke amplicons en werden nader bestudeerd. Om die reden werd het amplicon gesequenced en diverse overeenkomsten met bekende *S. aureus* sequenties werden gevonden. De meerderheid van de fragmenten vertoonden echter geen overeenkomst met bekende sequenties. Daarnaast werd van deze unieke amplicons het discriminerend vermogen getest aan de hand van hybridisatie experimenten met DNA van genetisch niet-verwante *S. aureus* stammen. Enkele fragmenten toonden het vermogen om een onderscheid te kunnen maken tussen verschillende stammen, op grond van de aan- of afwezigheid van het betrokken element in het genoom van de betrokken stafylokokken. Deze DNA fragmenten werden gebruikt als probes, om in een 5-cijferig typering systeem op nauwgezette wijze, epidemiologisch verwante en niet-verwante *S. aureus* stammen te onderscheiden. Een preliminair BT systeem voor *S. aureus* was hiermee ontworpen.

Genoom evolutie bij methicilline resistente *S. aureus* (MRSA) stammen werd bestudeerd en beschreven in **hoofdstuk 3**. Stammen met een lokaal, nationaal en wereldwijd verspreidingskarakter werden onderzocht. Het genotype van de stammen werd vastgesteld met behulp van diverse typering technieken, waarbij globaal het totale genoom werd gekarakteriseerd en door technieken, welke gericht waren op genetisch polymorfisme van specifieke genen. De verschillende gebruikte typering methoden lieten een hoge mate van heterogeniteit onder de epidemiologisch niet-verwante stammen zien. De diverse typering technieken vertoonden een variabel oplossend vermogen voor de

verschillende stammen binnen de drie stammencollecties, hetgeen gecorreleerd bleek te zijn aan de mate van geografische verspreiding van deze stammen. BT en de RAPD analyse waren beiden de meest geschikte methoden om verwantschap van stammen, met een gezamenlijke voorloper en een geringe mate van verspreiding, te analyseren.

In **hoofdstuk 4** wordt de nationale verspreiding van een MRSA kloon onder Turkse ziekenhuizen beschreven. Een collectie van 39 MRSA stammen, afkomstig van zes verschillende ziekenhuizen in Ankara en van één ziekenhuis gelegen in Bursa, Turkije, werden m.b.v. een aantal genotypering systemen geanalyseerd. In overeenstemming met de overige gebruikte typering systemen herkende BT de genetische homogeniteit van de onderzochte MRSA stammen. Hieruit mag worden geconcludeerd, dat een belangrijke MRSA kloon zich over een groot deel van Turkije heeft verspreid.

De toepasbaarheid van BT binnen de epidemiologie van *S. aureus* stammen met een bovine oorsprong wordt in **hoofdstuk 5** weergegeven. Hiervoor werden *S. aureus* stammen, geïsoleerd uit melk, genetisch gekarakteriseerd met behulp van de pulsed-field gel electroforese (PFGE) techniek en BT. In overeenstemming met elkaar werd de genetische verwantschap van de stammen met beide typering technieken opgehelderd. Bovendien werden de binaire types van de bovine stammen vergeleken met *S. aureus* stammen afkomstig van humane materialen. De diverse genotypen waren overwegend gastheer afhankelijk, maar sommige klonen hadden zowel een bovine als humaan verspreidingsgebied. Wij mogen concluderen, dat PFGE en BT met succes kunnen worden toegepast voor de genetische karakterisering van *S. aureus* stammen afkomstig uit koeienmelk, voor het ophelderen van eventueel gastheer gebonden populatie structuren, voor bronopsporing en tevens identificeren van verspreidingsroutes van de bovine *S. aureus* stammen.

In **hoofdstuk 6** wordt de bruikbaarheid van de naar een totaal van 15 uitgebreide stam-discriminerende DNA probes bestudeerd en gevalideerd volgens algemeen gehanteerde criteria voor het vaststellen van de prestaties van een moleculair typering systeem. Voor de validatie van BT werden *S. aureus* stammen, waarvan 69% MRSA, gebruikt. Alle DNA probes bleken stabiele epidemiologische markers. Het oplossend vermogen van BT overtrof dat van PFGE en van RAPD analyse. De reproduceerbaarheid van de test werd gemeten door een aantal stammen te gebruiken, die deel uit maakten van een aantal verschillende epidemische clusters. De BT analyse was conform de andere gebruikte genotypering technieken.

In **hoofdstuk 7** wordt een aangepast BT protocol beschreven. Het protocol is gebaseerd op een omgekeerde hybridisatie methode. De componenten van deze hybridisatie reactie zijn digoxigenine ULS® gelabeld genomisch DNA en 12 aan een membraanstrip vastgehechte DNA probes. Het doel van deze studie was het vereenvoudigen en het versnellen van de BT procedure. Een snelle en gestandaardiseerde nucleine zuur extractie procedure werd gebruikt voor het isoleren van DNA uit stafylokokken. Ongezuiverd, niet geamplificeerd genomisch stafylokokken DNA werd direct gelabeld met DIG-ULS®. De hybridisatie condities werden geoptimaliseerd en een

eenvoudige chromogene detectiemethode van de hybriden werd toegepast. Vijf identieke, vijf genetisch verwante en tien unieke *S. aureus* stammen werden als testpanel, gebruikt. BT bevestigde de uitslag van een eerder toegepaste genotypering techniek op deze stammencollectie.

De reproduceerbaarheid van de aangepaste BT werd onderzocht in een 2-traps multi-centrum studie (**hoofdstuk 8**). Allereerst werd als voorproef, in een Nederlandse multi-centrum studie (n=11), het binaire type van 10 genetisch unieke MRSA stammen in tweevoud vastgesteld. Als proces controle werd van tevoren het gelabeld DNA van een *S. aureus* stam meegezonden. Slechts drie laboratoria konden op adequate wijze alle stammen identificeren. Afwijkingen in de technische procedure resulteerden frequent in een foutieve beoordeling van de uitslagen. Het BT van de proces controle werd echter door alle deelnemende laboratoria juist vastgesteld. De slagingskans van het BT protocol was evenredig aan de moleculaire ervaring van het laboratorium personeel. Op grond van de resultaten verkregen met de nationale multi-centrum studie, konden we vaststellen dat het DNA extractie protocol diverse aanpassingen vereiste, om de kwaliteit en kwantiteit van het DNA te waarborgen.

Vervolgens namen zeven Europese centra deel aan een vervolgstudie met als doel, de reproduceerbaarheid van BT inclusief het verbeterde DNA isolatie protocol, te testen. Elk laboratorium werd gevraagd 10 verschillende DNA monsters (fase 1) afkomstig van de 10 genetisch unieke MRSA stammen uit de vorige studie, te testen. Bovendien werd hen verzocht het BT van deze 10 MRSA stammen vast te stellen, eventueel met behulp van hun eigen gestandaardiseerde DNA extractie methode (fase 2). Voor deze laatste studiefase werd een vooraf gelabeld DNA monster in de vorm van proces controle meegezonden. Alle centra, behalve één, waren in staat het binaire type van alle DNA monsters correct vast te stellen. Dit laatste centrum week van het voorgeschreven protocol af. Tevens werden ook het BT voor de proces controle door elk centrum op juiste wijze geïdentificeerd. Vijf van de zeven centra waren in staat de resultaten van BT voor alle MRSA stammen op juiste wijze te interpreteren. Drie van deze centra gebruikte hun eigen DNA isolatie protocol. Vanwege afwijkingen in de te volgen procedure, waren twee centra niet in staat de juiste BT resultaten van de MRSA stammen te produceren. Deze studie bewees, dat elk laboratorium al dan niet gebruik makend van een eigen DNA isolatie protocol, in staat is op reproduceerbare wijze BT resultaten te verkrijgen, mits er niet van het BT protocol wordt afgeweken.

Samenvattende conclusies. Het preliminaire BT, bestaande uit 5 DNA probes, was in staat op juiste wijze verwantschap tussen *S. aureus* stammen van humane en bovine afkomst aan te tonen. Na uitbreiding van het aantal DNA probes bleek BT een zeer discriminerend typering systeem te zijn. Vervolgens werd het BT protocol ontwikkeld tot een efficiënte techniek. De efficiëntie en reproduceerbaarheid van het vernieuwde BT protocol werd in een multi-centrum studie succesvol aangetoond.

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CURRICULUM VITAE

De auteur van dit proefschrift is geboren op 13 april 1954 te Rotterdam. Na de middelbare school is hij in 1971 begonnen met de hogere beroepsopleiding (A) tot microbiologisch analist aan het Van 't Hoff Instituut te Rotterdam. Sinds 1975 is hij werkzaam op de afdeling Bacteriologie van het Academisch Ziekenhuis Rotterdam Dijkzigt. Hiernaast volgde hij vanaf 1980 de avondopleiding voor hogere beroeps onderwijs (B) in de richting medische-biologie van het Dr. Struycken-Instituut te Etten-Leur, waar hij in 1983 zijn diploma behaalde. Vanaf 1994 was hij betrokken bij de ontwikkeling van moleculair-biologische technieken voor het karakteriseren van bacteriën onder begeleiding van Dr. W. Goessens en in een later stadium Dr. A. van Belkum. Gedurende deze periode werd de basis gelegd voor dit promotieonderzoek onder directe begeleiding van Dr.dr. A. van Belkum. Vanaf 1998 is het onderzoek uitgevoerd in samenwerking met Kreatech Diagnostics te Amsterdam, met als doel de ontwikkeling van een BT testkit voor productiedoeleinden. Na de promotie zal hij werkzaam blijven op de afdeling Medische Microbiologie & Infectieziekten van het Erasmus MC.

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