REVIEW

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Molecular Diagnostics of Clinically Important Staphylococci

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ABSTRACT. Bacterial species of the genus *Staphylococcus* known as important human and animal pathogens are the cause of a number of severe infectious diseases. Apart from the major pathogen *Staphylococcus aureus*, other species until recently considered to be nonpathogenic may also be involved in serious infections. Rapid and accurate identification of the disease-causing agent is therefore prerequisite for disease control and epidemiological surveillance. Modern methods for identification and typing of bacterial species are based on genome analysis and have many advantages compared to phenotypic methods. The genotypic methods currently used in molecular diagnostics of staphylococcal species, particularly of *S. aureus*, are reviewed. Attention is also paid to new molecular methods with the highest discriminatory power. Efforts made to achieve interlaboratory reproducibility of diagnostic methods are presented.

Abbreviations

EIA	enzyme immunoassay
HVR	hypervariable region
IS	insertion sequence
MIC	minimum inhibitory concentration
MLEE	multilocus enzyme electrophoresis
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecule
MSSA	methicillin-sensitive *Staphylococcus aureus*
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
REA	restriction endonuclease analysis
RFLP	restriction fragment length polymorphism
SCC*mec*	staphylococcal chromosome cassette methicillin-resistance island
SRFH	selective restriction fragment hybridization
SSCP	single-strand conformation polymorphism
VNTR	variable number of tandem repeat

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1 INTRODUCTION

Staphylococci are among the most widespread pathogenic and opportune pathogenic bacteria (Kloos *et al.* 1991; Holt *et al.* 1994). At present, 37 staphylococcal species are known, 9 of them including 2 subspecies, and 1 of them including 3 subspecies. Some taxa of the genus *Staphylococcus* are relevant to human medicine, others have been found only in other animals. Based on their capacity to produce free coagulase, staphylococci are traditionally divided into two groups: coagulase-positive species, considered to be pathogenic, and less dangerous coagulase-negative species (Table I).

Staphylococcus aureus, pathogenic to both humans and other animals, is the most relevant of the coagulase-positive species. It is a major clinical pathogen responsible for many serious infections among the general population and a leading cause of nosocomial infections. Non-human animals are the hosts of the non-*S. aureus* coagulase-positive species (Table I).

The complex cell-wall and extracellular proteins of *S. aureus* are constituted by multiple antigens and biologically active substances acting as pathogenicity factors: capsule (Sau *et al*. 1997), peptidoglycan, teichoic acids, agglutinogens, staphylococcal protein A, clumping factor (bound coagulase) (Ní Eidhin *et al*. 1998), other adhesins of the MSCRAMM family (Foster and Höök 1998), staphylocoagulase, staphylokinase, hyaluronidase, staphylococcal V8 proteinase (Coulter *et al*. 1998), hemolysins, leucocidins (Gravet *et al*. 1998), exfoliative toxins, enterotoxins, toxic shock syndrome toxin 1 (Dinges *et al*. 2000), epidermal cell differentiation inhibitor (Yamaguchi *et al*. 2001, 2002), and staphylococcal exotoxin-like proteins (Williams *et al*. 2000). Strains producing more exoproteins, such as exfoliative toxins or enterotoxins, are characterized by greater pathogenicity, pose a higher risk to humans, and cause more serious diseases. A central role in the pathogenesis of staphylococci has been assigned to the staphylococcal accessory gene regulator (*agr*), which increases the expression of many secreted virulence factors (Yarwood and Schlievert 2003).

The most clinically important coagulase-negative staphylococci are *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. sciuri* and *S. saprophyticus*. *S. epidermidis* is a typical opportunist pathogen known to cause a number of infections in weakened patients. Certain types of hospital-acquired infections (*e.g*., those associated with the use of catheters) are due to strains producing slime, *i.e*., an adherence factor. *S. haemolyticus* is the second most frequently isolated species of coagulase-negative staphylococci after *S. epidermidis*. It was detected in patients with infected wounds, urinary tract infections, endocarditis, and bacteremia. Interestingly, vancomycin resistance was reported in this species (Biavasco *et al*. 2000). *S. hominis* is the third most commonly isolated species whose multiresistant subspecies *S. homins* subsp. *novobiosepticus* (Kloos *et al*. 1998), mostly isolated from hemocultures or catheters, causes severe infections of the bloodstream (Petráš *et al.* 2002). *S. saprophyticus* is the causative agent of urinary tract infections. The species *S. sciuri* may cause wound infections and peritonitis and is considered to be the natural reservoir of the *mecA* gene – the methicillin resistance determinant (Wu *et al.* 1998; Marsou *et al*. 1999). *S. lugdunensis* and *S. schleiferi* subsp. *schleiferi* are opportunist pathogens, commonly associated with endocarditis, septicemia, and prosthetic device infections. Diseases caused by other species such as *S. cohnii*, *S. simulans*, or *S. warneri* are less fre-

Table I. Summary of known taxa of the genus *Staphylococcus* (modified according to Freney *et al*. 1999; Garrity *et al*. 2003; Kloos and Bannerman 1994)

Species/subspecies	Coagulase production	Host or source	Reference
S. arlettae	–	goats, poultry	Schleifer *et al*. 1984
S. aureus subsp. *anaerobius*	+	sheep	de la Fuenta *et al*. 1985
aureus	+	humans, animals, environment	Rosenbach 1884
S. auricularis	–	humans	Kloos and Schleifer 1983
S. capitis subsp. *capitis*	–	humans	Kloos and Schleifer 1975
ureolyticus	–	humans, some primates	Bannerman and Kloos 1991
S. caprae	–	humans, goats	Devriese *et al*. 1983
S. carnosus subsp. *carnosus*	–	meat products	Schleifer and Fischer 1982
utilis	–	foodstuffs	Probst *et al*. 1998
S. cohnii subsp. *cohnii*	–	humans	Schleifer and Kloos 1975
urealyticum	–	humans, animals	Kloos and Wolfshohl 1991
S. condimenti	–	soy sauce	Probst *et al*. 1998
S. delphini	+	dolphins	Varaldo *et al*. 1988
S. epidermidis	–	humans, animals, environment	Winslow and Winslow 1908; Schleifer and Kloos 1975
S. equorum subsp. *equorum*	–	horses, cattle	Schleifer *et al*. 1984
linens	–	surface-ripened cheese	Place *et al.* 2003
S. felis	–	cats	Igimi *et al*. 1989
S. fleurettii	–	goat milk cheese	Vernozy-Rozand *et al*. 2000
S. gallinarum	–	poultry, birds	Devriese *et al*. 1983
S. haemolyticus	–	humans, domestic animals, environment	Schleifer and Kloos 1975
S. hominis subsp. *hominis*	–	humans	Kloos and Schleifer 1975
novobiosepticus	–	humans	Kloos *et al*. 1998
S. hyicus	+[b]	animals, foodstuffs	Devriese *et al*. 1978
S. chromogenes	–	animals, milk	Devriese *et al*. 1978; Hájek *et al*. 1986
S. intermedius	+	mammals, birds, rarely humans	Hájek 1976
S. kloosii	–	wild animals	Schleifer *et al*. 1984
S. lentus	–	animals, rarely humans	Kloos *et al*. 1976; Schleifer *et al*. 1983
S. lugdunensis	–	humans	Freney *et al*. 1988
S. lutrae	+	otters	Foster *et al*. 1997
S. muscae	–	flies, pigs	Hájek *et al*. 1992
S. nepalensis	–	goats	Spergser *et al*. 2003
S. pasteuri	–	humans, animals, foodstuffs	Chesneau *et al*. 1993*b*
S. pettenkoferi[a]	–	humans	Trülzsch *et al*. 2002
S. piscifermentans	–	fermented fish	Tanasupawat *et al*. 1992
S. pulvereri[c]	–	humans, animals	Zakrzewska-Czerwinska *et al*. 1995; Švec *et al*. 2004
S. saccharolyticus	–	humans	Kilpper-Bälz and Schleifer 1981
S. saprophyticus subsp. *bovis*	–	animals	Hájek *et al*. 1996
saprophyticus	–	humans, mammals	Shaw *et al*. 1951
S. sciuri subsp. *carnaticus*	–	meat products	Kloos *et al*. 1997
rodentium	–	rodents, humans	Kloos *et al*. 1997
sciuri	–	humans, animals	Kloos *et al*. 1976
S. schleiferi subsp. *coagulans*	+	dogs	Igimi *et al*. 1990
schleiferi	–	humans	Freney *et al*. 1988
S. simulans	–	humans, mammals	Kloos and Schleifer 1975
S. succinus subsp. *casei*	–	surface-ripened cheese	Place *et al*. 2002
succinus	–	amber	Lambert *et al*. 1998
S. vitulinus	–	animals, foodstuffs	Webster *et al*. 1994
S. warneri	–	humans, primates	Kloos and Schleifer 1975
S. xylosus	–	humans, animals, environment	Schleifer and Kloos 1975

[a]The name is not valid yet. [b]Only some strains. [c]*S. pulvereri* reclassified as a later synonym of *S. vitulinus.*

quent (Iwantscheff *et al.* 1985; Waldon *et al.* 2002). Other coagulase-negative staphylococcal taxa are part of normal microflora in humans and animals, but may also play a role in some infections. Therefore, they are not to be considered automatically as contaminants since possibly implicated in severe and sometimes fatal diseases in weakened patients.

From the genetic standpoint, the species *S. aureus* and *S. epidermidis* are the best-studied (Table II). The genome of *S. aureus* consists of a single circular chromosome plus an assortment of variable accessory genetic elements: conjugative and nonconjugative plasmids, mobile IS and Tn elements, prophages and, recently described, genomic islands (Novick 1990, 2003; Młynarczyk *et al*. 1998; Hiramatsu 2001; Novick *et al*. 2001; Iandolo *et al.* 2002; Canchaya *et al.* 2003). Since individual strains of *S. aureus* differ from one another in the arrangement of chromosomal alleles and in the content of variable accessory genetic elements, DNA-based methods focusing on these differences have been introduced in genetic typing of *S. aureus* strains. Recently, the number of genomic sequences available in the DNA databases has expanded rapidly. Comparative genomics has been instrumental in cataloging genome-wide polymorphism, and has contributed greatly to the optimization of nucleic acid typing methods since the found variable genomic regions may be excellent typing targets. The practice of comparing gene sequences is well established, and predates the genomics era by many years. However, the application of this type of analysis to complete genomes greatly expands its utility and implications. DNA-based methods have contributed significantly to recent advances in the efficient tracking of the nosocomial and environmental spread of microbial pathogens. Not only has the application of novel technologies led to improved understanding of microbial epidemiology, but the concepts of population structure and dynamics of many of the medically important microorganisms have also advanced significantly. Genetic identification of microbes is also within the reach of clinical microbiology laboratory professionals, including those without specialized technology research interests because comercial assays are available (van Belkum 2003). DNA-based techniques, their advantages and disadvantages, comparison with phenotypic procedures, and use for identification and typing staphylococci will be discussed below.

Table II. Finished and incomplete staphylococcal genomes

Genome	Strain	GenBank accession no.	Size, bp	Institution	Reference
S. aureus	N315	NC_002745	2 813 641	*National Institute of Technology and Evaluation, Juntendo University*, Tokyo	Kuroda *et al*. 2001
	Mu50	NC_002758	2 878 040	*ditto*	Kuroda *et al*. 2001
	MW2	NC_003923	2 820 462	*ditto*	Baba *et al*. 2002
	COL	NC_002951	2 813 861	*The Institute for Genomic Research*, Rockville, USA	*not published*
	8325	NC_002954	2 821 361	*University of Oklahoma*, Tulsa, USA	*not published*
	MRSA 252	NC_002952	2 902 619	*Sanger Centre, Trinity College*, Hinxton, UK	Holden *et al.* 2004
	MSSA 476	NC_002953	2 799 802	*Sanger Centre, Trinity College*, Hinxton, UK	Holden *et al.* 2004
S. epidermidis	RP62A	NC_002976	2 655 392	*The Institute for Genomic Research*, Rockville, USA	*not published*
	ATCC 12228	NC_004461	2 499 279	*Chinese National Human Genome Center at Shanghai, Shanghai Medical University*	Zhang *et al.* 2003

2 COMPARISON OF GENOTYPIC AND PHENOTYPIC METHODS FOR TYPING OF STAPHYLOCOCCI

Bacterial typing is used to distinguish between strains (or clones) of a given species, which is of relevance to bacterial epidemiology since it permits one to recognize whether or not the causative agents of an outbreak of infection may originate from a single source.

The methods for typing of bacteria are based on their phenotypic and/or genotypic features. With staphylococci, the phenotypic methods include testing growth and biochemical characteristics (Kloos *et al.* 1991; Kloos and Bannerman 1999) by conventional or commercial identification sets, biotyping (Devriese

1984), phage typing (Blair and Williams 1961), antibiotic susceptibility testing (*NCCLS* 2003), whole-cell protein electrophoresis (Clink and Pennington 1987), zymotyping and MLEE (Tenover *et al*. 1994; Combe *et al*. 2000), cellular fatty acid analysis (Kotilainen *et al.* 1991), and serotyping and capsule typing (Karakawa *et al*. 1985; Fattom *et al*. 1992; Schlichting *et al*. 1993; Guidry *et al*. 1998).

Genotypic, or DNA-based methods focus on the characterization of chromosomal, plasmid, or total genomic DNA from the bacterial pathogen. The objectives are to assess relevant parameters of the genome and to detect polymorphism of DNA sequences by either direct (DNA sequencing) or indirect methods allowing generation of DNA fingerprints specific for isolates of distinct clonal lineages. The indirect genotypic methods include techniques either without DNA amplification (restriction analysis and DNA hybridization), or with DNA amplification by PCR and those identifying conformation polymorphisms of nucleic acids (Versalovic *et al*. 1993; Tenover *et al*. 1994, 1997; Vaneechoutte 1996; Struelens 1998). The resulting patterns can be evaluated either visually or by means of computer image analysis. At present emphasis is on the development of harmonized and validated tools with high discriminatory ability for typing of microorganisms and on interlaboratory reproducibility of the results as prerequisite for effective nosocomial infection control (*HARMONY* project).

Any typing system is rated based on the seven criteria defined below (Maslow *et al*. 1993; Maslow and Mulligan 1996; Struelens *et al*. 1996): (*i*) typeability refers to the proportion of isolates that can be scored in the typing system and assigned to a particular type; (*ii*) reproducibility refers to the ability of the typing system to assign the same type on repeat testing of the same strain; (*iii*) stability is the biological feature of clonally derived isolates to express constant markers over time and generations; (*iv*) discriminatory power of typing systems is defined as the average probability that different genotypes will be assigned to two unrelated strains in the population of a given genus; it is calculated by using the formula of Simpson's index of diversity as explained by Hunter and Gaston (1988) and Hunter (1990); (*v*) epidemiological concordance is the capacity of a typing system to correctly classify into the same clone all epidemiologically related isolates from a well-described outbreak; (*vi*) ease of interpretation, and (*vii*) ease of use are key issues for many techniques so that the techniques may be readily accepted by clinical microbiologists who generally lack the expertise that is required to discern differences between strains.

The genotypic compared to phenotypic methods have the advantage of being independent of the expression of specific genes under artificial assay conditions (*e.g*., laboratory media used) since the genotypic characters are relatively stable in nature compared to the phenotypic ones (biotype, serotype, antibiogram). The genotypic methods yield reproducible results regardless of variation in laboratory assay conditions; they are rapid, do not require *in vitro* cultivation and thus allow identification of fastidious or unculturable organisms. In contrast to the phenotypic methods, chromosomal DNA-based typing methods achieve typeability close to 100 %, since all bacteria contain DNA.

Discriminatory ability of genotypic compared to phenotypic methods is higher and thus allows typing of *S. aureus* strains that cannot be differentiated by bacteriophage typing, a phenotypic technique widely used for staphylococci. Moreover, the discriminatory power of the genotypic methods is not influenced by variability in expression of phenotypic characters, *e.g*., antimicrobial resistance shown in antibiograms (main typing tool in many hospital outbreaks).

3 GENOTYPIC METHODS USED FOR STAPHYLOCOCCI

3.1 Methods without DNA amplification

3.1.1 Restriction endonuclease analysis

Isolated total DNA is digested with a restriction enzyme, the originated fragments are separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Restriction endonucleases *Pst*I, *Eco*RI and *Pvu*II are used in staphylococci (Etienne *et al*. 1990; Haertl and Bandlow 1990). Restriction fragments in the size range 5–15 kb are separated by agarose gel electrophoresis; they represent a characteristic total genomic DNA fingerprint. Processing of record requires densitometric evaluation of gels and correlation comparison of densitometric curves (Matthews *et al.* 1992).

A method similar to REA is small fragment restriction endonuclease analysis (SF-REA), the only difference is in using polyacrylamide gel for separation of restriction fragments, into which only small-molar-mass DNA fragments (<1 kb) can enter. Restriction endonucleases such as *Eco*RI, *Bam*HI, and *Hin*dIII are used and the resulting fingerprint is visualized by silver staining. The SF-REA method was used for typing of *S. aureus* strains where it has a higher discriminatory ability than conventional REA on agarose gel (Haertl and Bandlow 1990; Tveten *et al*. 1991), comparable to that of PFGE (Haertl and Bandlow 1993).

3.1.2 Macrorestriction analysis

Total cellular DNA is isolated and digested with rare-cutting restriction enzyme, *e.g.*, *Sma*I or *Csp*I in *S. aureus*, so that commonly 10–30 macrorestriction fragments in the size range of 1–700 kb are generated, which are separated by PFGE. Isolates with identical restriction profiles are assigned the same macrorestriction type (PFGE type, pulsotype). Isolates that differ by one or two band shifts consistent with a single genetic event (*e.g.*, a point mutation resulting in the loss or gain of a restriction site, an insertion, a deletion, or a chromosomal inversion) are assigned a subtype, isolates with 3 and more such genetic differences are considered to be different types. According to these criteria, genetic relationships among isolates are determined for epidemiological purposes (Tenover *et al*. 1995). In comparison with other typing methods, PFGE has shown an equal or a greater discriminatory power (Maslow and Mulligan 1996), and superior reproducibility (Tenover *et al*. 1997). PFGE was used to determine interspecies and intraspecies genome variability in staphylococci (Snopková *et al*. 1994; Pantůček *et al*. 1996), and is the predominant method in use for studies of outbreaks and local epidemiology at the present time (Richardson and Reith 1993; Dominguez *et al*. 1994; Cox *et al*. 1995; Chang *et al*. 2000; Monsen *et al*. 2000; von Eiff *et al*. 2001); moreover, it has also been successfully applied to isolates separated by geographical location (Booth *et al*. 2001). Recently harmonized PFGE protocols for epidemiological typing of MRSA have been described (Murchan *et al*. 2003).

A variant of PFGE which utilizes a conventional electrophoretic chamber and periodically inverts the orientation of the electric field by 180° is designated as field inversion gel electrophoresis (FIGE). The technique provides comparable results like standard PFGE, but it is used less frequently. Better band separation was noted in the 50- to 200-kb range for FIGE, while PFGE revealed better resolution over 250 kb more reliably, including detection of some bands not seen on FIGE (Green *et al*. 1995). This method was utilized for epidemiological typing of staphylococci by Goering and Duensing (1990) and Goering and Winters (1992).

3.1.3 Selective restriction fragment hybridization

This method is used for detection of RFLP by Southern hybridization. Total cellular DNA is isolated and digested with a frequent-cutting restriction enzyme, *e.g.*, *Cla*I (Kreiswirth *et al.* 1993). The resulting fragments are separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with radioactively or nonradioactively labeled probes that target various genes or transposons, *e.g.*, *mecA*, Tn*554*, *agr* or *aacA–aphD* of *S. aureus* (Kreiswirth *et al.* 1993; Tenover *et al*. 1994). Synthetic oligonucleotides, *e.g.*, $(GTG)_5$-repeat suitable for all bacterial species, can also be used (Doll *et al*. 1993). The most important modifications of the SRFH method used for molecular typing of staphylococci are ribotyping, IS typing and prophage profiling.

Ribotyping is a special modification of RFLP typing using probes targeting 16S rRNA and/or 23S rRNA genes (*e.g.*, from *Escherichia coli* and *Bacillus subtilis* rRNA operons) (El Solh *et al*. 1990; Chesneau *et al*. 1992). The most often used restriction endonucleases are *Hin*dIII, *Cla*I (Tenover *et al*. 1994), *Eco*RI (Blumberg *et al*. 1992), *Eco*RV or *Kpn*I (Blanc *et al*. 1994). The number of polymorphic types is given not only by the difference in the number of restriction sites in rRNA genes and surrounding sequences, but also by the presence of more copies of *rrn* operons in the genome, which may differ from each other. This method exhibits excellent reproducibility and stability but its discriminatory power is lower than that of chromosomal DNA macrorestriction analysis with *Sma*I (Prévost *et al*. 1992) and comparable to MLEE (Struelens 1998). Ribotyping is suitable for identification of species and subspecies within the *Staphylococcus* genus (de Buyser *et al*. 1989, 1992) as well as for typing of strains of the same species (Hesselbarth and Schwarz 1995; Melter *et al*. 1999, 2003).

IS typing is a method resembling ribotyping. IS elements (insertion sequences; ISs) are transposable elements that do not carry any genetic information except that required for transposition. Although ISs do not code for any resistance, they are responsible for recombination and stabilization of some resistance genes and their presence is very important in the evolution of the bacterial genome. They are present at many sites on the chromosome, plasmids or composite transposons. Chromosomal DNA is cleaved by restriction endonucleases such as *Pst*I, *Bgl*II and probes targeting the core DNA sequence of an IS element (*e.g.*, IS*431* of *S. aureus*) are used (Tenover *et al*. 1994).

Prophage profiling is a novel SRFH method utilizing detection of prophages that are present in the chromosomes of most staphylococci. In *S. aureus*, temperate bacteriophages are members of the family *Siphoviridae* classified into 5 serological groups (A, B, C, F, L). There are great differences in the prophage content even between closely related *S. aureus* strains, the prophages of serogroups A, B and F being the most frequent to be detected. Prophage profiling utilizes hybridization of *S. aureus* genomic DNAs cleaved

by *Sma*I, *Eco*RI or *Hin*dIII with prophage-specific probes to detect the prophage species integrated in the genome (Borecká *et al*. 1996; Doškař *et al*. 2000; Pantůček *et al*. 2004).

3.1.4 Plasmid profiling and REA of plasmids (REAP)

Plasmids are variable components of many staphylococcal genomes, classified into 4 classes and 15 incompatibility groups and often responsible for antibiotic resistance (Novick 1990). For typing of multiresistant staphylococci the isolated intact plasmid DNA is separated by agarose gel electrophoresis and the number and sizes of plasmids are determined (Hall *et al*. 1989; Etienne *et al*. 1990; Tveten *et al*. 1991; Blumberg *et al*. 1992). This method is complementary to restriction analysis of plasmids in epidemiological studies (*see below*) allowing detection of possible plasmid loss or gain during the study. Since extrachromosomal DNA content shows considerable variation, plasmid analysis is suitable for the study of relatively recent epidemiological relationships, whereas analysis of chromosomal DNA reflects more reliably relationships over a longer period of time. Strains without plasmids are nontypeable.

In REAP the isolated plasmid DNA is digested separately with *Hin*dIII and *Eco*RI (combination utilized in *S. aureus* typing). The DNA digests obtained are electrophoresed through agarose gels and the patterns of restriction fragments produced for each enzyme are used to determine composite strain types (Tenover *et al*. 1994). This method can be complemented with specific gene detection using probes targeting for instance, genes encoding antibiotic resistance. Owing to the possible instability of plasmids, the method shows only moderate reproducibility (Tenover *et al*. 1994) and therefore an additional typing method is to be used for analysis (Hartstein *et al*. 1995).

3.1.5 Binary typing (BT)

BT of genomic DNA is based on hybridization of genomic DNA with a series of strain-differentiating DNA probes generated by arbitrarily primed PCR (van Leeuwen *et al*. 1996, 1998, 1999). The strain-specific DNA probes are generated as follows: hundreds of *S. aureus* DNA fingerprints obtained by AP-PCR analysis (*see Chapter 3.2.10*) are compared visually and unique strain-differentiating amplicons are selected and cloned. The cloned fragments are utilized for preparation of labeled DNA probes. The usefulness of 15 DNA probes was validated, according to generally accepted performance criteria for molecular typing systems. Hybridization with the given probe is scored 1, no hybridization is scored 0. The resulting binary code is transformed into a decimal number referred to as the binary type. This library probe genotyping system provides unambiguous, numerical clonal signatures. A BT procedure was technically simplified and accelerated by using reverse hybridization with immobilized probes, making it suitable for application in routine microbiology laboratories (van Leeuwen *et al*. 2001). An interlaboratory study proved the reproducibility of results provided that the standard BT protocol is followed to the letter, irrespective of the DNA isolation protocol used (van Leeuwen *et al*. 2002*a*). The BT discriminatory power is also superior to that of PFGE which was until recently regarded as the most discriminatory method.

3.2 Methods with DNA amplification

3.2.1 Gene-specific PCR

Amplification of conserved gene sequences by simplex PCR reaction is used for identification of staphylococcal species and their genotypes. In *S. aureus* the genes encoding 16S rRNA, factor A essential for methicillin resistance (*femA*), and staphylococcal thermonuclease (*nuc*) are frequently used for identification at the species level. Identification of coagulase-negative staphylococcal species important in hospital infection utilizes genes coding for 16S rRNA. The other genes used are listed in Tables III–VII.

3.2.2 PCR-RFLP typing

A target DNA sequence corresponding to a specific gene or genomic region is amplified at high stringency using primers annealing to its terminal conserved regions. The amplicon is cut with restriction endonuclease resulting in a specific RFLP pattern. This method is rapid, simple and reproducible but has shown only moderate discrimination. For instance, the coagulase gene cleaved with *Alu*I (Goh *et al*. 1992) or protein A-gene polymorphic region X cleaved with *Rsa*I were used in PCR-RFLP typing of *S. aureus* strains (Frénay *et al*. 1994). Other genes used are listed in Tables IV and V.

Table III. Genes and sequences used for molecular identification of *S. aureus* strains

Gene or sequence	Gene product or function	Detection method	Reference
16SrRNA	16S rRNA	PCR	Saruta *et al.* 1995; Schmitz *et al.* 1997, 1998*a*; Mason *et al.* 2001
		hybridization	Barry *et al.* 1990; Davis and Fuller 1991; Bentley *et al.* 1993; Freney *et al.* 1993; Greisen *et al.* 1994; Allaouchiche *et al.* 1996
23SrRNA	23S rRNA	PCR	Straub *et al.* 1999; Akineden *et al.* 2001; Stephan *et al.* 2001
		hybridization	Probst *et al.* 1998
aroA	5-enolpyruvylshikimate-3-phosphate synthase	PCR, hybridization	Marcos *et al.* 1999
clfA	clumping factor	PCR	Akineden *et al.* 2001; Mason *et al.* 2001
coa	staphylocoagulase	PCR	Schmitz *et al.* 1997, 1998*a*; Akineden *et al.* 2001
femA	cytoplasmic protein FemA	PCR	Ünal *et al.* 1992; Kobayashi *et al.* 1994
		multiplex PCR	Vannuffel *et al.* 1995, 1998
femB	cytoplasmic protein FemB	PCR	Kobayashi *et al.* 1994
		multiplex PCR	Towner *et al.* 1998; Jonas *et al.* 1999
gap	glyceraldehyde-3-phosphate dehydrogenase	PCR, PCR-RFLP, hybridization	Yugueros *et al.* 2000
hsp60 (*cpn60*)	60-kDa heat-shock protein HSP60 or chaperonin 60	hybridization	Goh *et al.* 1997
		sequencing	Goh *et al.*1996; Kwok *et al.* 1999; Kwok and Chow 2003
nuc	thermonuclease or heat-stable nuclease	PCR	Brakstad *et al.* 1992, 1995; Chesneau *et al.* 1993*a*; Brakstad and Maeland 1995; Barski *et al.* 1996; Khan *et al.* 1998
pnp	polynucleotide phosphorylase	hybridization	Fontana *et al.* 1999
rpoB	RNA polymerase β-subunit	sequencing	Drancourt and Raoult 2002
rRNA-spacer	16S–23S rRNA intergenic spacer sequences	RS-PCR	Saruta *et al.* 1997; Mendoza *et al.* 1998; Couto *et al.* 2001; Lee and Park 2001; Stephan *et al.* 2001
Sa2052 fragment	2 kb *Eco*RI subfragment of *S. aureus* 44 kb *Sma*I macrorestriction fragment	PCR, hybridization	Štěpán *et al.* 2001
Sa442 fragment	442 bp *Sau*3AI fragment of *S. aureus* genomic DNA	PCR, hybridization	Martineau *et al.* 1998; Klaassen *et al.* 2003
spa	staphylococcal protein A (two regions are detected: X-region and immunoglobulin G-binding region)	PCR	Akineden *et al.* 2001
tRNA-spacer	tRNA intergenic spacer sequences	PCR	Welsh and McClelland 1992; Maes *et al.* 1997
tuf	elongation factor Tu (EF-Tu)	PCR, hybridization	Martineau *et al.* 2001
		PCR-RFLP	Kontos *et al.* 2003

Table IV. Genes and sequences used for molecular typing of *S. aureus* strains

Gene or sequence	Gene product or function	Detection method	Reference
16SrRNA	16S rRNA (from *Escherichia coli* or *Bacillus subtilis*)	hybridization–ribotyping	de Buyser *et al.* 1989, 1992; El Solh *et al.* 1990; Blumberg *et al.* 1992; Chesneau *et al.* 1992; Tenover *et al.* 1994; Moore and Lindsay 2001
		sequencing-phylogeny	Takahashi *et al.* 1999
agr A, *agr* B, *agr* C, *agr* D	accessory gene regulator	sequencing	van Leeuwen *et al.* 2000; Jarraud *et al.* 2002; Dufour *et al.* 2002*a*
		nested PCR-RFLP	Papakyriacou *et al.* 2000; Mullarky *et al.* 2001; Gilot *et al.* 2002; Strommenger *et al.* 2004
		hybridization	Tenover *et al.* 1994; Moore and Lindsay 2001
aacA–aphD	acetyltransferase AAC(6′)/aminoglycoside phosphotransferase APH(2″)	hybridization	Tenover *et al.* 1994
aroA	5-enolpyruvylshikimate-3-phosphate synthase	PCR-RFLP	Marcos *et al.* 1999
cap5	capsule type 5	hybridization	Moore and Lindsay 2001
cap8	capsule type 8	hybridization	Moore and Lindsay 2001
ccrA, *ccrB*	cassette chromosome recombinase gene complex	multiplex PCR	Okuma *et al.* 2002 ; Aires de Sousa and de Lencastre 2003
coa	staphylocoagulase	PCR	Stephan *et al.* 2001
		PCR-RFLP	Goh *et al.* 1992; Schwarzkopf and Karch 1994; Tenover *et al.* 1994; Kobayashi *et al.* 1995; Lawrence *et al.* 1996; Hoefnagels-Schuermans *et al.* 1997; Hookey *et al.* 1998; Schmitz *et al.* 1998*c*; Annemüller *et al.* 1999; van Leeuwen *et al.* 1999; Wichelhaus *et al.* 2001; Montesinos *et al.* 2002
		hybridization	El-Adhami and Stewart 1998
dru	direct repeat units of 40 bp located in HVR adjacent to *mecA* gene	PCR	Tohda *et al.* 1997; Schmitz *et al.* 1998*c*; Witte *et al.* 2001; Nishi *et al.* 2002; Senna *et al.* 2002
		PCR-RFLP	Wichelhaus *et al.* 2001
		PCR-SSCP	Nishi *et al.* 1995
		sequencing	Nahvi *et al.* 2001
hsp60 (*cpn60*)	60-kDa heat-shock protein HSP60 or chaperonin 60	sequencing-phylogeny	Kwok *et al.* 1999; Kwok and Chow 2003
hsp70	heat shock protein 70	hybridization	Moore and Lindsay 2001
icaA	*N*-acetylglucosaminyltranferase for intercellular adhesin synthesis	PCR	Arciola *et al.* 2001*a*,*b*; Fowler *et al.* 2001; Vasudevan *et al.* 2003
		hybridization	Moore and Lindsay 2001
icaD	its co-expression with *icaA* is required for full slime synthesis	PCR	Arciola *et al.* 2001*a*,*b*; Vasudevan *et al.* 2003
IS*256*	*S. aureus* insertion sequence	hybridization	Morvan *et al.* 1997
IS*431*	*S. aureus* insertion sequence	hybridization	Tenover *et al.* 1994; Yoshida *et al.* 1997
map	major histocompatibility complex (MHC) class II analog protein (Map), also termed as extracellular adherence protein (Eap)	hybridization	Smeltzer *et al.* 1997

continued

mecA	low-affinity penicillin-binding protein PBP 2′, methicillin resistance	hybridization	Kreiswirth *et al.* 1993, 1995; de Lencastre *et al.* 1994, 1996*a*,*b*, 1997; Dominguez *et al.* 1994; Tenover *et al.* 1994; Santos-Sanches *et al.* 1995, 1996; Teixeira *et al.* 1996; Soares *et al.* 1997; Mato *et al.* 1998; Roberts *et al.* 1998; Melter *et al.* 1999; Sá-Leão *et al.* 1999; Oliveira *et al.* 2001*b*; Aires de Sousa *et al.* 2003
		PCR-RFLP	Wu *et al.* 1998
mecI	repressor protein of the *mecA* gene	PCR	van Leeuwen *et al.* 1999
		hybridization	Suzuki *et al.* 1993; de Lencastre *et al.* 1996*a*; Mato *et al.* 1998
		sequencing	Watson *et al.* 2003
mecR1	membrane signal protein controlling the expression of methicillin resistance	PCR	van Leeuwen *et al.* 1999
		hybridization	Suzuki *et al.* 1993
Prophages	sequences of resident prophages classified into phage species 3A, 11, 77 and 187	multiplex PCR	Pantůček *et al.* 2002
		hybridization	Doškař *et al.* 2000
rpoB	RNA polymerase β-subunit	sequencing-phylogeny	Drancourt and Raoult 2002
rRNA-spacer	16S–23S rRNA intergenic spacer sequences	PCR	Dolzani *et al.* 1994; Gürtler and Barrie 1995; Kumari *et al.* 1997; Saruta *et al.* 1997; Schmitz *et al.*1998*c*; Annemüller *et al.* 1999
sarA	staphylococcal accessory regulator A	hybridization	Moore and Lindsay 2001
sbi	IgG and β_2-glycoprotein I (or apolipoprotein H) binding protein	hybridization	Moore and Lindsay 2001
SCC*mec*	staphylococcal chromosome cassette methicillin-resistance	multiplex PCR-type assignment	Okuma *et al.* 2002; Oliveira and de Lencastre 2002; Ma *et al.* 2002; Aires de Sousa and de Lencastre 2003
spa	staphylococcal protein A	PCR	Schmitz *et al.* 1998*c*; Annemüller *et al.* 1999; Stephan *et al.* 2001; Montesinos *et al.* 2002
		PCR-RFLP	Frénay *et al.* 1994; Hoefnagels-Schuermans *et al.* 1997; van Leeuwen *et al.* 1999; Wichelhaus *et al.* 2001; Sabat *et al.* 2003
		sequencing	Frénay *et al.* 1996; Kobayashi *et al.* 1999; Shopsin *et al.* 1999; Oliveira *et al.* 2001*b*
		hybridization	Moore and Lindsay 2001
splB	serine proteinase-like B protein	hybridization	Moore and Lindsay 2001
ssp	serine proteinase V8	PCR-RFLP	Sabat *et al.* 2003
		hybridization	Moore and Lindsay 2001
Tn*554*	*S. aureus* transposon	hybridization	Kreiswirth *et al.* 1993, 1995; de Lencastre *et al.* 1994, 1996*a*,*b*, 1997; Dominguez *et al.* 1994; Tenover *et al.* 1994; Santos-Sanches *et al.* 1995, 1996; Teixeira *et al.* 1996; Soares *et al.* 1997; Mato *et al.* 1998; Roberts *et al.* 1998; Melter *et al.* 1999; Sá-Leão *et al.* 1999; Oliveira *et al.* 2001*b*; Aires de Sousa *et al.* 2003

Table V. Genes coding for surface protein adhesins used for molecular typing of *S. aureus* strains

Gene	Gene product	Detection method	Reference
clfA	clumping factor A	PCR	Stephan *et al.* 2001
		PCR-RFLP	Sabat *et al.* 2003
		multiplex PCR	Tristan *et al.* 2003
		hybridization	Smeltzer *et al.* 1997
clfB	clumping factor B	PCR-RFLP	Sabat *et al.* 2003
		multiplex PCR	Tristan *et al.* 2003
cna	collagen-binding protein	PCR	Montanaro *et al.* 1999; van Leeuwen *et al.* 1999
		multiplex PCR	Tristan *et al.* 2003
		hybridization	Smeltzer *et al.* 1996, 1997; van Leeuwen *et al.* 1999; Moore and Lindsay 2001
ebpS	elastin-binding protein	multiplex PCR	Tristan *et al.* 2003
		hybridization	Smeltzer *et al.* 1997
eno	laminin-binding protein	multiplex PCR	Tristan *et al.* 2003
efb (*fib*)	extracellular fibrinogen-binding protein	PCR	Bodén Wästfelt and Flock 1995
		multiplex PCR	Tristan *et al.* 2003
		hybridization	Bodén Wästfelt and Flock 1995; Smeltzer *et al.* 1997; Moore and Lindsay 2001
fbpA	fibrinogen-binding protein	hybridization	Smeltzer *et al.* 1997
fnbA, *fnbB*	fibronectin-binding proteins A and B	multiplex PCR	Tristan *et al.* 2003
		hybridization	Smeltzer *et al.* 1996, 1997; Moore and Lindsay 2001
sdr	serine-aspartate (SD) repeat-containing proteins	PCR-RFLP	Sabat *et al.* 2003

Table VI. Staphylococcal toxin-encoding genes detected by hybridization, PCR and multiplex PCR

Gene	Gene product	Detection method	Reference
edin-A, edin-B, edin-C	epidermal cell-differentiation inhibitors EDIN-A, EDIN-B, EDIN-C	hybridization	Gravet *et al.* 2001
		PCR	Jarraud *et al.* 2002; Yamaguchi *et al.* 2002
		PCR, sequencing	Czech *et al.* 2001
eta	exfoliative toxin A	hybridization	Rifai *et al.* 1989; Moore and Lindsay 2001
		PCR	Jarraud *et al.* 1999, 2001, 2002; Akineden *et al.* 2001
		multiplex PCR	Becker *et al.* 1998; Mehrotra *et al.* 2000
etb	exfoliative toxin B	hybridization	Rifai *et al.* 1989; Moore and Lindsay 2001
		PCR	Jarraud *et al.* 1999, 2001, 2002; Akineden *et al.* 2001
		multiplex PCR	Becker *et al.* 1998; Mehrotra *et al.* 2000
etd	exfoliative toxin D	PCR	Yamaguchi *et al.* 2002
hla	α toxin (α-hemolysin)	hybridization	Moore and Lindsay 2001
		PCR	Jarraud *et al.* 2002
hlb	β toxin (β-hemolysin; sphingomyelinase C)	hybridization	Smeltzer *et al.* 1996; Moore and Lindsay 2001
		PCR	Jarraud *et al.* 2002
hlg	γ toxin (γ-hemolysin)	hybridization	Prévost *et al.* 1995; Moore and Lindsay 2001
		PCR	Jarraud *et al.* 2002
hld	δ toxin (δ-hemolysin)	PCR	Jarraud *et al.* 2002
lukE–lukD	LukE–LukD, class S and class F leucocidin components	hybridization	Moore and Lindsay 2001
		PCR	Dufour *et al.* 2002*b*; Jarraud *et al.* 2002
lukM–lukF′–PV	LukM–LukF′–PV, class S and class F leucocidin components	PCR	Jarraud *et al.* 2002
lukS-PV–*lukF*–PV	Panton–Valentine leucocidin, class S and class F components	hybridization	Prévost *et al.* 1995
		PCR	Lina *et al.* 1999*a*; Dufour *et al.* 2002*b*; Jarraud *et al.* 2002
set1–set11	staphylococcal exotoxin-like proteins SET1 to SET11	hybridization	Gravet *et al.* 2001
		PCR	Fitzgerald *et al.* 2003
tst	toxic shock syndrome toxin 1	hybridization	Neill *et al.* 1990; Shimaoka *et al.* 1996; Moore and Lindsay 2001
		PCR	Saulnier *et al.* 1997; Jarraud *et al.* 1999, 2001, 2002; McLauchlin *et al.* 2000; Akineden *et al.* 2001
		multiplex PCR	Schumacher-Perdreau *et al.* 1995; Becker *et al.* 1998; Schmitz *et al.* 1998*b*; Monday and Bohach 1999; Mehrotra *et al.* 2000
Classical enterotoxin types			
sea	enterotoxin A	hybridization	Neill *et al.* 1990; Gravet *et al.* 1999, 2001; Moore and Lindsay 2001
seb	B	PCR	Tsen and Chen 1992; Jarraud *et al.* 1999, 2001, 2002; McLauchlin *et al.* 2000; Akineden *et al.* 2001; Atanassova *et al.* 2001; Fueyo *et al.* 2001; Hazariwala *et al.* 2002

sec	C	multiplex PCR	Schumacher-Perdreau *et al.* 1995; Becker *et al.* 1998; Schmitz *et al.* 1998*b*; Monday and Bohach 1999; Mehrotra *et al.* 2000; Sharma *et al.* 2000; Omoe *et al.* 2002
sed	D		
see	E	multiplex QPCR	Letertre *et al.* 2003
	New enterotoxin types		
seg	enterotoxin G	hybridization	Gravet *et al.* 2001; Moore and Lindsay 2001
seh	H	PCR	Jarraud *et al.* 1999, 2001, 2002; McLauchlin *et al.* 2000; Akineden *et al.* 2001; Fueyo *et al.* 2001; Rosec and Gigaud 2002
sei	I		
sej	J	multiplex PCR	Monday and Bohach 1999; Omoe *et al.* 2002; Becker *et al.* 2003
sek	K	multiplex QPCR	Letertre *et al.* 2003
sel	L		
sem	M		
sen	N		
seo	O		
sep	P	DNA microarray	commercial DNA arrays (ExpressChip, Mergen, San Leandro), Kuroda *et al.* 2001
seq	Q	DNA microarray	Yarwood *et al.* 2002
ser	R	hybridization	Omoe *et al.* 2003

3.2.3 *Multiplex PCR assay*

The multiplex PCR assay allows simultaneous amplification of several genes in one reaction mixture. It is used for detection of genes encoding various types of MSCRAMMs (Table V), toxins (Table VI), or antibiotic resistance in staphylococci (Table VII). Recently multiplex PCR assays for identification of prophages (Pantůček *et al.* 2004) and of the SCC*mec* element structural types and their variants were developed (Oliveira and de Lencastre 2002).

3.2.4 *Quantitative PCR (QPCR)*

The real-time quantitative PCR system is based on the detection and quantitation of a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product in a reaction. General methods for the quantitative detection of the amplicon, DNA-binding agents and fluorescent probes including TaqMan® technology, molecular Beacons™ and fluorescence resonance energy transfer (FRET) probes have been used in the study of staphylococci. Real-time PCR has recently been used for identification of *S. aureus* species (Yang *et al.* 2002; Palomares *et al.* 2003) and 15 species of coagulase-negative staphylococci (Edwards *et al.* 2001). Rapid QPCR procedures were developed for identification of methicillin resistance (Reischl *et al.* 2000; Elsayed *et al.* 2003; Francois *et al.* 2003) and fluoroquinolone resistance genes (Lapierre *et al.* 2003). Reverse transcription-quantitative PCR (RT-QPCR) enables quantitation of bacterial transcripts and study of antimicrobial resistance and virulence gene expression *in vivo* (Goerke *et al.* 2001).

3.2.5 *Ribosome spacer PCR (RS-PCR) or PCR ribotyping*

RS-PCR is a method based on the highly polymorphic nature of the 16S–23S rRNA internal transcribed spacer sequences which are amplified by primers containing conserved sequences from the adjacent 16S and 23S rRNA genes (Barry *et al.* 1991). RS-PCR allows detection of a wide range of length and sequence polymorphisms at the genus and species levels. Analysis of these intergenic spacer sequences proved useful in differentiating between species (Jensen *et al.* 1993; Mendoza *et al.* 1998; Couto *et al.* 2001) or strains (Gürtler and Barrie 1995) within the genus *Staphylococcus*. RS-PCR is

Table VII. Genes associated with *S. aureus* antibiotic resistance detected by hybridization, PCR and multiplex PCR

Gene	Gene product and its function	Detection method	Reference
aacA–aphD	aminoglycoside acetyltransferase AAC(6′)/phosphotransferase APH(2″); aminoglycoside resistance	PCR, multiplex PCR	Martineau *et al.* 2000*a*,*b*
aadC, *aphA*3	aminoglycoside adenyltransferase ANT(4′,4″), and phosphotransferase APH(3′)III; aminoglycoside resistance	PCR	Vanhoof *et al.* 1994
blaZ	β-lactamase, penicillin resistance	multiplex PCR	Martineau *et al.* 2000*a*,*b*
ermA	erythromycin rRNA methylase (methyltransferase 23S rRNA); macrolide, lincosamide and streptogramin B resistance	hybridization	Khan *et al.* 2000
		multiplex PCR	Khan *et al.* 2000; Martineau *et al.* 2000*a*,*b*; Strommenger *et al.* 2003
		PCR	Lina *et al.* 1999*b*
ermB	erythromycin rRNA methylase (methyltransferase 23S rRNA); macrolide, lincosamide and streptogramin B resistance	multiplex PCR	Martineau *et al.* 2000*a*,*b*
		PCR	Lina *et al.* 1999*b*
ermC	erythromycin rRNA methylase (methyltransferase 23S rRNA); macrolide, lincosamide and streptogramin B resistance	hybridization	Khan *et al.* 2000
		multiplex PCR	Khan *et al.* 2000; Martineau *et al.* 2000*a*,*b*; Strommenger *et al.* 2003
		PCR	Lina *et al.* 1999*b*
grlA, *B*	mutations in genes coding for subunits of DNA topoisomerase IV; fluoroquinolone resistance	PCR and sequencing	Takahashi *et al.* 1998; Messina *et al.*2001
gyrA, *B*	mutations in genes coding for subunits of DNA gyrase; fluoroquinolone resistance	PCR and sequencing	Goswitz *et al.* 1992; Takahashi *et al.* 1998; Messina *et al.*2001
		PCR-SSCP	Tokue *et al.* 1994; Takenouchi *et al.* 1995
ileS-2	isoleucyl tRNA synthetase 2 (non-sensitive to mupirocin); mupirocin resistance	multiplex PCR	Anthony *et al.* 1999; Nunes *et al.* 1999; Pérez-Roth *et al.* 2002
linA′	lincosaminide *O*-nucleotidyltransferase; lincosamide resistance	PCR	Lina *et al.* 1999*b*
mecA	low-affinity penicillin-binding protein PBP 2′; methicillin resistance	hybridization	Duck *et al.* 1990; Figueiredo *et al.* 1991; Tokue *et al.* 1992; Shimaoka *et al.* 1994; Gorelov *et al.* 1996; Bekkaoui *et al.* 1999; Moore and Lindsay 2001; Smyth *et al.* 2001; van Leeuwen *et al.* 2002*b*
		PCR	Murakami *et al.* 1991; Predari *et al.* 1991; Tokue *et al.* 1992; Geha *et al.* 1994; Del Vecchio *et al.* 1995; Gorelov *et al.* 1996; Wallet *et al.* 1996; van Leeuwen *et al.* 1999, 2002*b*
		multiplex PCR (mostly combination with detection of *S. aureus* specific genes)	Ünal *et al.* 1992; Kobayashi *et al.* 1994; Brakstad and Maeland 1995; Vannuffel *et al.* 1995, 1998; Barski *et al.* 1996; Schmitz *et al.* 1997, 1998*a*; Towner *et al.* 1998; Jonas *et al.* 1999; Nunes *et al.* 1999; Martineau *et al.* 2000*a*,*b*; Mehrotra *et al.* 2000; Mason *et al.* 2001; Smyth *et al.* 2001; Pérez-Roth *et al.* 2002
msrA	ATP-dependent efflux pump; partial macrolide-streptogramin B (MS) resistance	PCR, multiplex PCR	Lina *et al.* 1999*b*; Martineau *et al.* 2000*a*, *b*
rpoB	mutations in RNA polymerase β-subunit gene; rifampicin resistance	PCR and sequencing	Aubry-Damon *et al.* 1998
tet (many forms)	tetracycline efflux pump; tetracycline resistance	multiplex PCR	Warsa *et al.* 1996; Ng *et al.* 2001; Strommenger *et al.* 2003
vanA	D-alanyl-D-lactate ligase; high-level vancomycin resistance	multiplex PCR	Ramos-Trujillo *et al.* 2003
vatA, *B*, *C*	acetyltransferase genes; streptogramin A resistance	PCR, multiplex PCR	Lina *et al.* 1999*b*; Strommenger *et al.* 2003
vga, *vgaB*	ATP-binding proteins probably involved in active efflux of the A compounds of virginiamycin-like antibiotics	PCR	Lina *et al.* 1999*b*

a rapid, inexpensive technique that is highly reproducible and almost as discriminatory as PFGE for typing of MRSA isolates (Kumari *et al*. 1997).

3.2.6 tDNA intergenic spacer length polymorphism (tDNA-ILP)

tDNA-ILP is another internal transcribed spacer-PCR method based on the different distances separating conserved tRNA genes in staphylococcal species (Welsh and McClelland 1992). Since tRNA genes contain sequence motifs that are highly conserved among the eubacteria, consensus tRNA gene primers can be designed to explore intergenic length polymorphisms between species (Maes *et al*. 1997). Particular species are distinguished according to the number and size of generated PCR products.

3.2.7 Multiple-locus variable number of tandem repeat (VNTR) analysis (MLVA)

MLVA represents simultaneous PCR-RFLP analysis of several loci containing VNTR. The *sdr*, *clfA*, *clfB*, *ssp* and *spa* genes are used for *S. aureus*. MLVA is equivalent to PFGE in discriminatory power and reproducibility (Sabat *et al*. 2003).

3.2.8 PCR-gene single-strand conformation polymorphism typing (PCR-SSCP)

This typing method is based on conformation polymorphism of single strands derived from polymorphic sequences. The PCR product (100–450 kb) is denatured by formamide or by heating, and the resulting single strands are separated on a nondenaturing polyacrylamide gel by electrophoresis. Polymorphic alleles (a single nucleotide difference is sufficient) give rise to single strands with different conformation which influences their electrophoretic mobility. In *S. aureus*, the method was used for analysis of bacterial DNA gyrase A subunit gene (*gyrA*), in which point mutations are associated with fluoroquinolone resistance (Tokue *et al*. 1994; Takenouchi *et al*. 1995). The HVRs adjacent to *mecA* in MRSA and methicillin-resistant coagulase-negative staphylococci were compared by using SSCP, too (Nishi *et al*. 1995). PCR-SSCP is also suitable for molecular identification of bacteria based on of the 16S rRNA gene (Widjojoatmodjo *et al*. 1995).

3.2.9 Cleavase fragment length polymorphism (CFLP)

CFLP analysis is a rarely used method combining PCR-RFLP and PCR-SSCP. The 5´-end-labeled PCR products (350-bp fragment of the 16S rRNA gene in staphylococci) are denatured by heating, then cooled and the created hairpin-like structures are partially digested by the *Cleavase*I® enzyme at the junctions between duplexed and single-stranded regions. Separation of the resulting fragments by electrophoresis produces characteristic banding patterns which clearly differentiate bacteria to the genus level and in some cases to the species and strain levels (Brow *et al*. 1996).

3.2.10 Arbitrarily primed PCR (AP-PCR, RAPD)

AP-PCR is based on the use of short oligonucleotide primers (8–12 oligomers) with an arbitrary sequence under low-stringency PCR conditions (Welsh and McClelland 1990; Williams *et al*. 1990). Information about the target sequence is not needed. A strain-specific DNA-fingerprint comprised of amplified DNA fragments of various sizes is obtained. The discriminatory power varies according to the number and sequences of the arbitrary primers and amplification conditions, but is inferior to that of PFGE in typing of MRSA strains (Saulnier *et al*. 1993). In spite of its attractive efficiency, AP-PCR typing suffers from problems in reproducibility and from the lack of consensus rules for interpretation of results (Struelens *et al*. 1993; van Belkum *et al*. 1995; Maslow and Mulligan 1996). A number of technical factors need to be strictly standardized for optimal reproducibility (Grundmann *et al*. 1997). The patterns obtained are evaluated preferably visually rather than by using gel analysis software (Burr and Pepper 1997). The method is adequate for rapid comparative typing but less suitable for library typing in surveillance programs.

3.2.11 Repetitive PCR (rep-PCR)

The PCR amplification of genomic regions between interspersed repeated DNA sequences (referred to as "rep") is based on different positions of DNA elements on the chromosome and various distances separating them, depending on the particular bacterial strain and species. Repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences belong to these repetitive elements (Versalovic *et al*. 1991). Other repetitive elements used in rep-PCR are IS*256* (IS sequence of *S. aureus*) (Deplano *et al*. 1997), RepMP3 (from *Mycoplasma pneumoniae*) usable for Gram-positive bacteria (Del Vecchio *et al*. 1995, van der Zee *et al*. 1999), and BOX (from *Streptococcus pneumoniae*), which seems to be an excellent tool for the identification of *S. epidermidis* (Wieser and Busse 2000).

Opposing PCR primers matching these sequences yield various numbers of differently long fragments under high-stringency amplification conditions depending on the bacterial strain, from which DNA originates; however, low-stringency PCR conditions and different primer combinations are used in practice (van Belkum *et al*. 1993). A method based on RFLP analysis of rep-PCR-amplicons derived from the newly described staphylococcal repetitive element named STAR (Cramton *et al.* 2000) was developed for typing MRSA strains (Quelle *et al.* 2003). Compared to AP-PCR, rep-PCR produces fewer amplified DNA fragments, but possesses good discriminatory power and its major advantage is higher reproducibility. Nevertheless, the rep-PCR methods produce an array of DNA fragments showing interlaboratory variability thus leading to the problem of data comparability (Deplano *et al*. 2000). A particular improvement to this approach is fluorophore-enhanced rep-PCR (FERP) which uses primers labeled at their 5′ ends with a fluorescent compound, separation of PCR products in a nondenaturing polyacrylamide gel, which is scanned by a laser scanning unit enabling digital processing of DNA fingerprints (Del Vecchio *et al*. 1995; Versalovic *et al*. 1995). Another modification is automated laser fluorescence analysis (ALFA) also using 5′ fluorescently labeled primers with PCR products being separated in a denaturing polyacrylamide gel and resolved in an automated DNA sequencer, which moderately improves the resolution and interlaboratory reproducibility of rep-PCR patterns compared to agarose gel analysis (Deplano *et al*. 2000).

3.2.12 Amplified fragment length polymorphism (AFLP) and infrequent-restriction-site amplification (IRS-PCR)

AFLP (Vos *et al*. 1995) and IRS-PCR (Mazurek *et al*. 1996) are innovative PCR-based strategies, which also appear to offer high resolution and good reproducibility. The techniques involve three steps: (*i*) total restriction of the genomic DNA and ligation of specially designed oligonucleotide adapters, (*ii*) selective amplification of sets of restriction fragments, and (*iii*) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of their nucleotide sequence. The AFLP technique provides a novel and very powerful DNA fingerprinting tool for DNAs of any origin or complexity. This method has a high discriminatory power (Sloos *et al*. 1998, 2000) and enables species and strain identification of staphylococci (Velappan *et al*. 2001). The genetic background of toxin-producing *S. aureus* strains was determined by means of AFLP analysis (Jarraud *et al*. 2002).

3.3 Methods based on direct DNA sequence determination

3.3.1 DNA sequencing

Nucleic acid sequencing is a direct method for the detection of DNA sequence polymorphism. It is the most sensitive method with the highest discriminatory power. Its disadvantage is the time required and the cost of the procedure currently limiting its use.

In staphylococci, like in other bacterial species, most often ribosomal RNA genes (16S or 23S rRNA) are sequenced for identification purposes (Sasaki *et al*. 1997). Nucleotide sequences of these genes are highly conserved and, therefore, make it possible to determine phylogenetic relationships (Ludwig and Schleifer 1992; Takahashi *et al*. 1999).

In MRSA, DNA sequencing was used for studying staphylococcal protein-A gene (*spaA*) polymorphism (Frénay *et al*. 1996). The number of 24-bp repeats designated as short sequence repeats (SSRs) in the region X of *spa* has been related to the dissemination potential of MRSA. Higher numbers of repeats associate with higher epidemic capability because a longer X region results in a better exposure of the Fc-binding region of protein A, thereby facilitating colonization of the host surfaces (Frénay *et al*. 1994; Walker *et al*. 1998). *spaA* typing, *i.e.*, the determination of the organization of the repeat units in the X region give reproducible, unambiguous, and easily interpreted results and seems to be adequate for outbreak investigations but should be complemented with other techniques in long-term surveillance or in studies comparing distant clonal lineages (Oliveira *et al*. 2001*b*).

Other examples of direct sequencing are listed in Tables III, IV, VI and VII.

3.3.2 Multilocus sequence typing (MLST)

MLST provides a new approach to molecular epidemiology that can identify and track the global spread of virulent or antibiotic-resistant isolates of bacterial pathogens using the Internet (Enright and Spratt 1999). MLST is a highly discriminatory method of characterizing bacterial isolates on the basis of the se-

quences of ≈450 bp internal fragments of 7 housekeeping genes (Maiden *et al*. 1998): carbamate kinase (EC 2.7.2.2; *arcC*), shikimate 5-dehydrogenase (EC 1.1.1.25; *aroE*), glycerol kinase (EC 2.7.1.30; *glpF*), guanylate kinase (EC 2.7.4.8; *gmk*), phosphate acetyltransferase (EC 2.3.1.8; *pta*), triose-phosphate isomerase (EC 5.3.1.1; *tpi*) and acetyl-CoA *C*-acetyltransferase (EC 2.3.1.9; *yqiL*).

The sequences obtained are assigned allele numbers following comparison of the DNA sequence with the sequences of previously typed strains by using the MLST website (**`www.mlst.net`**). The allele numbers at each of the seven loci define the allelic profile or sequence type (ST). Novel alleles and STs not found on the MLST website are confirmed by repeating both the PCR and sequencing.

MLST was utilized for identifying the MRSA and MSSA clones among isolates from patients with serious community- and hospital-acquired infections (Enright *et al*. 2000). This method is suitable for studying both the evolution of MRSA pandemic clones (Oliveira *et al*. 2001*a*, 2002*a*) and local epidemiology (Huygens *et al.* 2002; Aires de Sousa *et al.* 2003; Melter *et al.* 2003), and is similar in discriminatory power to PFGE (Peacock *et al*. 2002). MLST provides generally highly reproducible and comparable results. However, it has the disadvantage of being expensive and technically demanding. Therefore, there are attempts to detect sequence polymorphisms of the 7 loci by other methods instead of DNA sequencing, *e.g.*, DGGE (*see Chapter 3.4.1*) (Gürtler *et al.* 2002), PCR-RFLP (Diep *et al.* 2003) and DNA microarrays (*see Chapter 3.4.2*) (van Leeuwen *et al.* 2003).

3.4 Innovative high-throughput molecular methods

3.4.1 Denaturing gradient gel electrophoresis (DGGE)

The separation of DNA molecules by DGGE is based on melting properties of DNA. In the DGGE system, DNA fragments which have the optimum size of 150–1000 bp are electrophoresed through a polyacrylamide gel that contains a linear denaturing gradient. The molecules form branched structures that have retarded mobility in the gel matrix. If the gradient conditions are chosen properly, DGGE allows a sequence-specific separation of a mixture of DNA PCR-products of the same length, differing by a single-base branching. In staphylococci the DGGE method was used for detection of *mec* gene variants in MRSA isolates (Ahmadinejad *et al.* 1998) and detection of mutations in the VS2 region of the 16S–23S rRNA intergenic spacer region (Gürtler *et al.* 2001). To obviate the need for multilocus sequencing, a DGGE method was developed for MLST (Gürtler *et al.* 2002).

3.4.2 DNA microarrays

DNA microarrays provide considerable acceleration in molecular biology assays. Oligonucleotides anchored on the membrane or on the glass slide hybridize with labeled PCR products or with cDNA. The result is recorded by an apparatus – the scan is digitized and analyzed using appropriate software. The high-density microarrays containing thousands of oligonucleotides constituting ≈90 % of the *S. aureus* genome are used in the study of deletions in open reading frames (Somerville *et al*. 2002) and regulation of the virulence factor production (Dunman *et al*. 2001). The microarray containing 16S rDNA probes can be used for the species identification of bacteria (Wilson *et al*. 2002). The low-density microarrays containing tens of oligonucleotides were used for identification of 5 staphylococcal species and methicillin resistance (using the probes *femA* and *mecA*) (Hamels *et al*. 2001). The microarray containing 23S rDNA probes was used for identification of pathogenic bacteria including *S. aureus* (Anthony *et al*. 2000). Microelectronic chip arrays exist too, simplifying the process of bacterial identification. They enable anchored *in situ* amplification, discrimination and detection on the same platform (Westin *et al*. 2001).

3.4.3 Fluorescence in situ *hybridization with peptide nucleic acid probes (FISH PNA)*

Peptide nucleic acid (PNA) is an analogue of DNA in which the backbone is made of repeating *N*-(2-aminoethyl) glycine units linked by peptide bonds. The different bases are linked to the backbone by methylene carbonyl linkages. PNA mimics the behavior of DNA and obeys Watson–Crick base-pairing rules for hybridization to complementary nucleic acid targets. Due to their uncharged, neutral backbones, PNA probes exhibit favorable hybridization characteristics, such as high specificities, strong affinities, and rapid kinetics, resulting in improved hybridization to highly structured targets such as rRNA. In addition, the relatively hydrophobic character of PNA compared to that of DNA oligonucleotides enables PNA probes to penetrate the hydrophobic cell wall of bacteria. A fluorescence *in situ* hybridization (FISH) method for identification of *S. aureus* directly from positive blood cultures without cultivation and biotyping has been described (Kempf *et al.* 2000). A novel technique that combines the unique performance characteristics of

PNA probes targeting rRNA with FISH has recently been applied for rapid and specific identification of bacteria including *S. aureus* (Oliveira *et al.* 2002*b*, 2003).

3.4.4 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS is a technique for creating ionized gas-phase molecules accelerated in an electric field, followed by a flight through a vacuum chamber to a detector. Specific characteristics for the samples under study are their molar mass. Recently, this technique was experimentally applied to intact bacterial cells or specimens prepared using extraction procedures. The method is used for identification of the *S. aureus* species and differentiation between MSSA and MRSA strains (Edwards-Jones *et al.* 2000; Du *et al.* 2002). Only small discrepancies were found between the MALDI-TOF MS and PCR results. Furthermore, the combination of 1-D protein gel electrophoresis and MALDI-TOF MS could be a good tool for a rapid analysis of pathogenic factors in clinical isolates (Bernardo *et al.* 2002).

4 GENES AND PROBES USED FOR DIAGNOSTICS OF STAPHYLOCOCCI

4.1 Genes and sequences used in identification of staphylococcal species

Most staphylococcal, clinically important, species are ordinarily identified by PCR or hybridization methods targeting variable 16S rRNA gene regions (*for references see* Table III). Another frequently used method for rapid species identification is 16S–23S intergenic spacer length polymorphism estimated by RS-PCR, which is also used in some other genera of Gram-positive cocci (Drahovská *et al.* 2002). DNA sequences from the highly conserved and ubiquitous *hsp60* gene offer a convenient and accurate tool for species-specific identification and phylogenetic analysis of staphylococci; however, they are used to a lesser extent. In strains exhibiting atypical features, ribotyping or 16S rRNA PCR in conjunction with automated sequencing can be used for their identification at the species level.

The detection of a number of genes and genomic sequences has been used for identification of *S. aureus* strains (Table III). Hybridization, PCR or DNA sequencing and comparison of the determined sequences with those available in international databases are used for this purpose. Direct application to a variety of clinical specimens for rapid diagnosis of *S. aureus* infection is PCR detection of the thermonuclease gene (*nuc*), used routinely in clinical microbiology laboratory.

Multiresistant staphylococci are usually identified by testing with multiplex PCR assays or hybridization for species identification and detection of associated antibiotic resistance genes and their essential factors. Both plasmid-carried and chromosomal genes are detected (Table VII).

4.2 Specific genes and sequences used in typing of *S. aureus* strains

Analysis of a number of genes and genomic sequences listed in Tables IV and V is used for typing of *S. aureus* strains. Gene polymorphism was analyzed using hybridization, PCR (presence and/or absence of the product, various product lengths), PCR-RFLP, identification of DNA sequences and their comparison with the known sequences of the international database, which can also be used for phylogenetic analysis. The ability to produce toxins is associated with the presence of particular genes, which can also be detected by multiplex PCR. *S. aureus* toxin genes detectable by hybridization and PCR methods are summarized in Table VI.

4.3 Identification and typing of methicillin-resistant *S. aureus* strains

Methicillin (or oxacillin) resistance was identified in staphylococci shortly after the introduction of methicillin in clinical practice. The first European outbreaks of infection caused by MRSA were reported in Denmark and the UK in the early 1960s (Jevons 1961). MRSA has spread worldwide in both the hospital and community environment, particularly among patients of chronic care facilities, to pose a serious therapeutic problem (Ayliffe 1997; Chambers 1997).

The key genetic component of methicillin resistance, the *mecA* determinant encoding low-affinity penicillin-binding protein PBP 2′ or PBP 2a, is not native to *S. aureus*, and was acquired from an unknown heterologous source some time before the first reported appearance of MRSA isolates in clinical specimens (Crisóstomo *et al*. 2001). The *mecA* structural gene is carried by a newly described variable genetic element

SCC*mec* that belongs to the general category of chromosomal islands (Katayama *et al*. 2000). Integration of this element into the chromosome converts drug-sensitive *S. aureus* into the notorious hospital pathogen methicillin-resistant *S. aureus* (MRSA), which is resistant to practically all β-lactam antibiotics (Hiramatsu *et al*. 2001). This element not only carries the methicillin resistance gene but is also a highly preferred site for the insertion of other accessory genetic elements including plasmids, transposons, and IS elements, varying consequently in size from ≈20 to >60 kb. A remarkable feature of these other insertions is that they carry only resistance genes (Novick *et al*. 2001). After acquiring an SCC*mec* element, MRSA undergoes several mutational events and evolves into the most difficult-to-treat pathogen in hospitals, against which all extant antibiotics including vancomycin are ineffective (Hiramatsu *et al*. 2001).

In total, 4 types of *mec* elements were described in MRSA (Ito *et al*. 2001; Oliveira *et al*. 2001*a*; Ma *et al*. 2002). The SCC*mec* types I–IV are defined by the particular combination of two parts, *viz*., a *ccr* complex (types 1, 2, and 3) plus the *mec* complex (classes A and B). The *ccr* complex contains a pair of conserved recombination genes, *ccrA* and *ccrB*, that belong to the invertase and/or resolvase family; the entire sequence of class A *mecA* complex is *mecI–mecR1–mecA*–IS*431*, that of class B *mecA* complex is IS*1272*–Δ*mecR1-mecA*-IS*431* (resulting from a deletion in regulatory genes and integration of an insertion sequence). SCC*mec*–I comprises type 1 *ccr* complex and class B *mecA* complex, SCC*mec*–II comprises type 2 *ccr* complex, class A *mecA* complex, and furthermore the pUB110 integrated plasmid, SCC*mec*–III comprises type 3 *ccr* complex, class A *mecA* complex, and furthermore the pT181 and pI258 integrated plasmids, SCC*mec*–IV comprises type 2 *ccr* complex and class B *mecA* complex and do not harbor any antibiotic resistance genes except *mecA*. There are minor variants of SCC*mec* types I, III, and IV designated as IA, IIIA, IIIB, and IVA (Oliveira *et al*. 2001*a*). Compared to type I, the IA variant includes a 1-kb deletion in the HVR and nearly always the pUB110 integrated plasmid; the pT181 integrated plasmid is absent in the IIIA variant compared to type III, both pT181 and pI258 integrated plasmids are absent in the IIIB variant; the IVA variant includes in contrast to type IV an additional pUB110 integrated plasmid. Recently, within type IV SCC*mec* several subtypes were distinguished which differ from one another by regions upstream from the *ccr* gene complex (Ito *et al.* 2003). The findings reported here indicate that the *mecA* gene region is considerably polymorphic, and the downstream region of the *mecA* gene is the most variable (Oliveira *et al*. 2000), which is of relevance to epidemiological typing.

Many phenotypic and genotypic methods for identifying MRSA are used in medical microbiology laboratories. The phenotypic methods include the disc diffusion test with an oxacillin disc, cultivation on selective agar media (*e.g.*, on mannitol salt agar containing oxacillin or in oxacillin broth in multi-well plate by using an automated identification system like the ATB Staph system (*bioMérieux*, France) measuring turbidity, or the BBL Crystal MRSA ID system (*Becton Dickinson*, USA) measuring fluorescence of an oxygen-sensitive fluorescent indicator in UV light during bacterial growth), and MIC determination of oxacillin by using an agar dilution method.

S. aureus MIC breakpoints recommended for oxacillin are: susceptible ≤2 μg/mL and resistant ≥4 μg/mL; nevertheless, for methicillin-resistant coagulase-negative staphylococci lower the oxacillin breakpoints, *i.e*. ≤0.25 μg/mL and ≥0.5 μg/mL, were recently recommended by the *National Committee for Clinical Laboratory Standards* (*NCCLS* 1999; Tenover *et al*. 1999). Another method is the MRSA screen latex test identifying the *mecA* gene product PBP 2a by agglutination with sensitized latex particles (Wallet *et al*. 1996; Smyth *et al*. 2001; van Leeuwen *et al*. 2002*b*). While the latex test provides results within 15 min, other tests need at least 1 d to be readable; moreover, the drawbacks of phenotypic methods, such as errors in detection of methicillin resistance, especially in coagulase-negative staphylococci, are well known (Hájek *et al*. 2002).

Genotypic methods proving the presence of the low-affinity penicillin-binding protein PBP 2′ gene (*mecA*) by using hybridization with a labeled probe, or PCR (Table VII), are preferable for detection of methicillin resistance in staphylococci. Hybridization can also be performed benefiting from the use of cycling probe technology (CPT) with a 5′-fluorescein labeled, 3′-biotinylated DNA–RNA–DNA chimeric *mecA* probe, run at a constant temperature (Duck *et al*. 1990; Bekkaoui *et al*. 1999; van Leeuwen *et al*. 2002*b*). The probe anneals to the target DNA sequence in *mecA*-carrying strains, and RNAase H cuts the RNA portion in the probe, allowing two DNA fragments to dissociate from the target sequence. Detection of the uncleaved probe from strains lacking the *mecA* gene is performed by EIA in a streptavidin-coated multi-well microtiter plate format, where the probe is captured by its 3′-biotinylated end, and subsequently detected by horse-radish-peroxidase conjugated anti-fluorescein antibody resulting in blue color development. The method is rapid, reliable, and takes only 1½–2 h.

Discrimination of MRSA from the other methicillin-resistant staphylococcal species can be performed by simultaneous detection of the *mecA* gene and one of the following *S. aureus* specific genes: *femA*, *femB*, *coa*, 16S rDNA, *nuc*, *clfA* by using either hybridization or multiplex PCR (Table III).

After the introduction of genotyping methods based on DNA analysis, increasing significantly the resolution of epidemiological typing, the *Sma*I macrorestriction analysis has become the most-trusted epidemiologic marker system for MRSA typing, since it is highly discriminative, stable, and reproducible (Prévost *et al*. 1992; Struelens *et al*. 1992, 1993; Schlichting *et al*. 1993; Tenover *et al*. 1994; Nada *et al*. 1996; Na'was *et al*. 1998; Schmitz *et al*. 1998*c*; Olive and Bean 1999).

The combination of 3 molecular typing techniques (hybridization of *Cla*I fragments of digested genomic DNA resolved by agarose gel electrophoresis with *mecA* probe, hybridization of the same fragments with Tn*554* probe, and PFGE of *Sma*I macrorestriction fragments of genomic DNA) is frequently used for typing of MRSA, yielding a *Cla*I–*mecA*::*Cla*I–Tn*554*::*Sma*I-PFGE pattern. A particular notation can read for instance III::B::A. Patterns are interpreted according to Kreiswirth *et al*. (1993), de Lencastre *et al*. (1994), Tenover *et al*. (1995) and Chung *et al*. (2000). This typing method is widely used for tracing the clonal origin, and the spread of MRSA throughout the hospital wards, and within large territories up to continents in epidemiological surveillance (Dominguez *et al*. 1994; de Lencastre *et al*. 1994, 1996*a*,*b*, 1997; Santos-Sanches *et al*. 1995, 1996; Teixeira *et al*. 1996; Soares *et al*. 1997; Mato *et al*. 1998; Roberts *et al*. 1998; Melter *et al*. 1999; Sá-Leão *et al*. 1999; Oliveira *et al*. 2001*b*; Aires de Sousa *et al*. 2003). Previous surveillance studies carried out primarily in Southern and Eastern Europe, Latin America, and the United States, have characterized >3000 MRSA hospital isolates of which ≈70 % can be classified into 5 clonal types showing extensive geographic spread. Three clonal types (Iberian, Brazilian, and Hungarian clones) share a common or closely related genetic background A, being the same as the background of the earliest European isolates of MRSA from the UK and Denmark. The Pediatric and New York and/or Japan clones belong to the completely different genetic background B (Oliveira *et al*. 2001*a*).

*Cla*I–*mecA*::*Cla*I–Tn*554*::*Sma*I-PFGE typing can be supported by hybridization with a *mecI* regulatory gene probe (de Lencastre *et al*. 1996*a*; Mato *et al*. 1998). However, *Cla*I–*mecA*::*Cla*I–Tn*554* typing alone was used in previous studies (Figueiredo *et al*. 1991; Kreiswirth *et al*. 1993, 1995), while the combination of the two methods was used for validation of binary typing (van Leeuwen *et al*. 1999). Tenover *et al.* (1994) utilized a combination *Cla*I–*mecA*::*Cla*I–Tn*554mec*::*Cla*I–*agr*::*Cla*I–*aacA–aphD* for comparison of traditional and molecular methods for *S. aureus* typing. Single probes (Suzuki *et al*. 1993) or primers (van Leeuwen *et al*. 1999) for detection of *mec* regulatory genes *mecI* and *mecR1* are used less commonly. IS typing using an IS*431* probe has also been reported to be useful in MRSA epidemiology (Tenover *et al*. 1994). The combined use of ribotyping, PFGE typing and IS*431* typing showed a high discriminatory power (Yoshida *et al*. 1997). The IS*256* probe can also be used (Morvan *et al*. 1997) to improve analysis of *Sma*I-PFGE macrorestriction profiles.

Ryffel *et al.* (1991) described *dru* (direct repeat units of 40 bp) sequences in MRSA composing a HVR in the *mec* region between the *mecA* gene and IS*431*. Genotyping with *mec*-HVR, which amplifies VNTR of *dru* sequences can be used to improve MRSA typing (Nishi *et al.* 1995, 2002; Tohda *et al*. 1997; Schmitz *et al*. 1998*c*; Witte *et al*. 2001), *dru* sequencing is a useful tool for tracking methicillin-resistant lineages of staphylococci (Nahvi *et al*. 2001). *mec*-HVR-PCR is rapid, easy to perform, reproducible and has the ability to obtain an unambiguous positive result for each isolate analyzed. This technique shows a discriminatory power inferior to that of PFGE and therefore is less reliable for MRSA typing than PFGE (Senna *et al*. 2002).

Another typing method using the polymorphic *mec* element (Oliveira *et al*. 2000) is based on assaying for the presence or absence of variable or mobile elements previously shown to be associated with the *mecA* region: Δ*mecR1* (a regulator gene of methicillin resistance containing a 170 bp deletion), a distinct HVR allele from that described by Ryffel *et al.* (1991), integrated resistance plasmids pUB110, pI258 and pT181, an insertion sequence IS*256*, and Ins*117* (117-bp sequence of unknown function flanked by two 15-bp direct repeats). This method can be used to distinguish community-acquired (epidemic) and health care facility-acquired (endemic) MRSA isolates, creating a genetic background (Huygens *et al*. 2002). Unlike the well-identified community-acquired isolates the more diverse health-care facility-acquired strains need to be typed by a background genotyping method, such as PFGE, if the relationship between isolates is to be determined accurately (Huygens *et al*. 2002). A multiplex PCR strategy was developed for the characterization of MRSA clonal types and the rapid tentative identification of the *mec* element structural variants (Oliveira and de Lencastre 2002; Aires de Sousa *et al*. 2003). The SCC*mec* types are specifically associated with different pandemic clones: types I and III with isolates of genetic background A, type II with isolates of genetic background B, and type IV related to type I, with isolates of genetic background A as well as B (Oliveira *et al*. 2001*a*). All community-acquired MRSA strains were originally described as being SCC*mec* type IV and not carrying any resistance genes other than *mecA* (Hiramatsu *et al*. 2001). However, isolates that contain either the pT181 plasmid (encoding tetracycline and mercury resistance) or the pUB110 plasmid (encoding kanamycin and neomycin resistance) were found (Huygens *et al*. 2002).

4.4 Glycopeptide-intermediate-resistant *S. aureus*

Recent reports indicate that MRSA has continued to mutate and has developed intermediate resistance to vancomycin (Hiramatsu 2001). During the last several years vancomycin-intermediate resistant *S. aureus* strains (VISA) were isolated from patients with underlying diseases whose long-term vancomycin treatment apparently failed. Although isolates with homogeneous resistance to vancomycin (MIC = 8 μg/mL) continue to be rare, there are increasing reports of strains showing heteroresistance, often with vancomycin MIC in the 1–4 μg/mL range which have been isolated from many areas of the world, and are associated with infections that are potentially refractory to vancomycin therapy (Walsh and Howe 2002). Since many VISA isolates also have been resistant to teicoplanin, the term glycopeptide-intermediate *S. aureus* (GISA) is more appropriate (Linares 2001). Vancomycin resistance has been reported in clinical isolates of both *S. aureus* and coagulase-negative staphylococci.

GISA isolates are identified on the basis of their phenotypic properties (Midolo *et al.* 2003). The mechanism of resistance involves a complex reorganization of cell-wall metabolism, leading to a grossly thickened cell wall with reduced peptidoglycan cross-linking. The diversity of changes documented in the cell walls of GISA isolates is evidence that a single genetic or biochemical change cannot account for resistance in all isolates described to date (Boyle-Vavra *et al*. 2001). However, in 2002 a clinical isolate of *S. aureus* with high-level resistance to vancomycin (MIC = 1 024 μg/mL) was isolated in USA. This isolate harbored a 57.9-kb multiresistance conjugative plasmid within which Tn*1546* (*vanA*) was integrated. Genetic analyses suggest that the transfer of vancomycin resistance to a methicillin-resistant *S. aureus* occurred *in vivo* by interspecies transfer of Tn*1546* from a co-isolate of *Enterococcus faecalis* (Weigel *et al.* 2003).

The emerging threat of widespread vancomycin resistance poses a serious public health concern given the fact that vancomycin has long been the preferred treatment of antibiotic-resistant Gram-positive organisms (Srinivasan *et al*. 2002).

5 CONCLUSIONS

DNA-based typing methods are in principle universally applicable to all bacteria. Dissimilarities are only in the use of particular restriction endonucleases, hybridization probes (except ribotyping utilizing universal 16S or 23S rRNA probes), and PCR primers. However, primers derived from conserved sequences of rRNA and tRNA genes, those for rep-PCR derived from universally occurring bacterial repetitive elements, those for AP-PCR arbitrary in their sequences, and those for AFLP and IRS-PCR that are complementary to special oligonucleotide adapters and to a restriction site are applicable universally. The most often used method for typing staphylococci is PFGE; however, AP-PCR and rep-PCR are also used frequently. These methods were evaluated with respect to inter-laboratory reproducibility and data transferability. The PFGE method appears to be the golden standard and might possibly be developed into a surveillance typing system. The disadvantage arising from the generation of banding patterns, which are often difficult to reproduce and to be interpreted without bias, can be bypassed by binary typing and methods based on nucleotide sequence detection (*i.e*. direct sequencing and MLST). Moreover, both binary typing and MLST can be easily performed by using DNA microarrays. Binary typing is currently best suited for immediate implementation in the routine laboratory, but it is anticipated that sequence-based methodologies will prevail in the future. Other novel methods applicable to identification and typing of staphylococci are QPCR, DNA-microarrays and PNA probes. As they are technically demanding and expensive, these methods are unlikely to be routinely used in the foreseeable future.

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