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Comparison of Phage Typing and DNA Fingerprinting by Polymerase Chain Reaction for Discrimination of Methicillin-Resistant *Staphylococcus aureus* Strains

ALEX VAN BELKUM,[1]* RENÉ BAX,[1] PAUL PEERBOOMS,[2] WIL H. F. GOESSENS,[3] NAN VAN LEEUWEN,[4] AND WIM G. V. QUINT[1,3]

Diagnostic Centre SSDZ, P.O. Box 5010, 2600 GA Delft,[1] Municipal Health Service Amsterdam, 1018 WT Amsterdam,[2] Academic Hospital Dijkzigt, Erasmus University Rotterdam, 3015 GD Rotterdam,[3] and National Institute of Public Health and Environmental Protection, 8720 BA Bilthoven,[4] The Netherlands

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A typing procedure for methicillin-resistant *Staphylococcus aureus* (MRSA) based on the polymerase chain reaction (PCR) amplification of both *mecA* sequences and variable DNA sequences as present in the prokaryotic genome has been developed. Two primers based on the sequences of DNA repeats as discovered in gram-negative members of the family *Enterobacteriaceae* allow detection of variable regions in the genome of a gram-positive bacterium such as *S. aureus*, as does a newly described arbitrary primer. This procedure, enabling the detection of 23 different genotypes in a collection of 48 MRSA isolates, was validated by comparisons with phage typing studies. It appeared that within the same group of isolates only 13 different phagovars could be identified. Combination of the results from both phage typing and genotyping allowed the discrimination of 34 of 48 isolates. However, depending on the primer-variable complexity of the PCR fingerprints, which could also be modulated by combination of PCR primers, clear homologies between the groups defined by either phage typing or fingerprinting were observed. An analysis of an MRSA outbreak in a geriatric institution showed a collection of genetically homogeneous isolates. In agreement with phage typing, PCR fingerprinting revealed the identical natures of the MRSA strains isolated from all patients.

Several biochemical and molecular biological parameters can be used to distinguish isolates of a given genus or species. Many procedures for typing of methicillin-resistant *Staphylococcus aureus* (MRSA) epidemics have been developed (2, 6, 14). Besides the traditional phage typing method for *S. aureus*, several alternative techniques have been applied in comparative, epidemiological studies. Among these are serotyping (13), capsular typing (18), electrophoretic analysis of protein content (5), pulsed-field gel electrophoresis (10), restriction fragment length polymorphism analyses (10), and ribotyping (14). In addition to epidemiological studies of clinical isolates, attention has been focused on the development of adequate detection assays for MRSA. The polymerase chain reaction (PCR) has proven to be an adequate means for the detection and typing of a multitude of microorganisms (8, 9, 11, 16). As a consequence, several PCR and probe assays specific for the *mecA* gene of *S. aureus*, encoding penicillin-binding protein 2a, have been evaluated (1, 7, 12, 19, 20). PCR at present allows sensitive detection of MRSA in clinical specimens (12). For typing of microorganisms in particular, random amplified polymorphic DNA analysis is more and more frequently used in order to discriminate between individual species or isolates of a given species (4, 23, 24). As an alternative to the arbitrary random amplified polymorphic DNA analysis, it is feasible to amplify known polymorphic regions within DNA molecules in a way which gives rise to variable DNA fingerprints. Repeated DNA motifs are especially amenable to this approach. PCR fingerprinting with primers aiming at, for instance, eukaryotic simple sequences or telomeric repeats (21) or prokaryotic repetitive extragenic palindromes and enterobacterial repetitive intergenic consensus sequences (ERIC) (22) proves to be of value in the identification of genetically similar but not identical organisms or isolates of a given species.

In this study, traditional phage typing of MRSA was compared with newly developed PCR fingerprinting procedures aiming both at the *mecA* gene, as a determinant for methicillin resistance, and at variable DNA sequences. This comparison allows the evaluation of both procedures, and their practical applicability is discussed.

MATERIALS AND METHODS

Bacterial strains. Forty-eight randomly isolated clinical strains of MRSA were provided by the Laboratory for Medical Microbiology of the Academic Hospital Dijkzigt in Rotterdam, The Netherlands. These strains were identified by using standardized microbiological procedures. All strains were identified as *S. aureus* on the basis of colony morphology, color, and positive catalase and coagulase tests. Phenotypic methicillin resistance was determined by the disk diffusion assay as described. Briefly, isolated colonies from an overnight plate are suspended in saline to a turbidity of a McFarland standard of 0.5. To inoculate the Mueller-Hinton agar plates (Oxoid CM 337), a cotton swab is dipped into the standardized suspension and streaked in three directions over the entire plate surface to obtain a uniform inoculum. Subsequently, a disk containing 5 μg of methicillin (Oxoid) is applied. Plates are inverted and incubated for a full 24 h at a temperature of 30°C. Plates are examined, and the diameters of the inhibition zones are measured by a template. Colonies with inhibition zones with

* Corresponding author.

TABLE 1. DNA primers evaluated with respect to their potential applicability in PCR-mediated genetic typing of MRSA isolates

Primer no.	Primer sequence (5′ to 3′)	Primer type	Reference or source
2650	AATCGGGCTG	Arbitrary	4
961	AATCGGGCNG	Arbitrary	This study
2651	CAATCGCCGT	Arbitrary	4
963	CAATCGCCNT	Arbitrary	This study
1026	TACATTCGAGGACCCCTAAGTG	Arbitrary	This study
1116	TCACGATGCA	Arbitrary	24
934	AAAATCGATGGTAAAGGTTGGC	*mecA* gene 1	12
935	AGTTCTGCAGTACCGGATTTGC	*mecA* gene 2	12
1064	TTGACGAAAATTGAATGGAGAATAGACGTTAAAATGAATC	*S. aureus dru* sequence	15
1065	CTAAGTAAAATTGCAGATAAGAGGTAAGTTAAAAGCAGTT	*S. aureus* inverted *dru* sequence	15
1204	ATGTAAGCTCCTGGGGATTCAC	ERIC1R	22
1014	AAGTAAGTGACTGGGGTGAGCG	ERIC2	22
933	$(AGC)_7$	Simple sequence motif	21

a diameter smaller than 17 mm are regarded as resistant, or, in the case of growth of separate colonies in the inhibition zone, strains are also regarded as methicillin resistant. The Municipal Health Service of Amsterdam provided a collection of MRSA isolates from a single outbreak in a geriatric nursing house and harboring multiple isolates from individual patients. Variations were in the body site from which the sample was taken and the date of sampling. Also, a single isolate displaying a phage type which strongly deviated from the ones obtained for the Dijkzigt isolates was provided. This strain was analyzed together with those of the outbreak. All strains were grown as monocultures in brain heart infusion broth and incubated in a rotary shaker at 37°C for 18 h.

DNA isolation. Since the isolation of *S. aureus* is hampered by the rigidity of the bacterial cell wall, lysostaphin treatment is generally included in the preparation of lysates. Combinations of several extraction procedures were tested for efficiency of DNA isolation. A sample of 500 μl of the cultures was preincubated with either 15 μg of lysostaphin or 25 μg of proteinase K and 10 μl of 10% sodium dodecyl sulfate (SDS) to digest the bacterial cell wall. Incubation took place for 1 h at 37°C. The subsequent isolation and purification of DNA were performed either with 4 M guanidine-isothiocyanate and Celite according to previously described procedures (3) or by phenol extraction followed by ethanol precipitation (17). DNA concentrations were estimated by gel electrophoresis through 1% agarose gels in 40 mM Tris-acetate (pH 7.8)–2 mM EDTA, staining with ethidium bromide, and comparison with samples containing a known amount of DNA (17). Proteinase K-SDS treatment, combined with guanidine-isothiocyanate extraction, generated DNA preparations of the highest quality.

Phage typing. Analysis of MRSA phage susceptibility was performed at the Dutch National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). An international set of typing phages and an additional set of Dutch phages as well as MRSA-specific phages were used for the establishment of the phagovar.

Primers and primer synthesis. Oligodeoxyribonucleotide primers were synthesized on an Applied Biosystems model 381A DNA synthesizer and used without any further purification. DNA concentrations were determined by measuring the A_{260} on a Uvicam model PU 8720 spectrophotometer. A list of the primers used is presented in Table 1.

PCR. The reaction mixtures consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, and 0.1% Triton X-100. Deoxyribonucleotide triphosphates were used at a final concentration of 0.2 mM. For each reaction, 0.5 U *Taq* DNA polymerase (Sphaero-Q, Leiden, The Netherlands) was added. PCR conditions consisted of 40 cycles of consecutive denaturation, annealing, and DNA chain extension (1 min at 94°C, 1 min at 25°C, and 2 min at 74°C) in a Biomed model 60 thermocycler. Southern blots and probe hybridization were performed as described before (12).

RESULTS

Conventional *S. aureus* strain typing. For 48 MRSA strains, the presence of the methicillin resistance gene complex was confirmed by *mecA*-specific PCR followed by Southern blot hybridization of the PCR products (data not shown) (reference 12). Phage typing identified 13 phagovars of MRSAs. The number of strains belonging to each phagovar varied from 1 to 9. Most of the strains used could not be typed by application of the international phage sets. Phages of international group III showed some reactivity only with isolates 9 and 23. For all other strains, the use of a collection of Dutch MRSA-specific phages led to discrimination as shown in Table 2. The phages used (and descriptions of those phages) are available on request.

PCR fingerprinting. An increase in the ratio between genomic DNA and PCR primers resulted in an overall synthesis of longer amplimers; smaller fragments tended to disappear from the DNA banding patterns. Probably, the higher the primer concentration is, the more primers may anneal to less specific target sequences, causing more and smaller PCR fragments to be generated. The best interpretation of the DNA fingerprint results was achieved with 5 ng of DNA and a total of 100 pmol of primers (either single or in combination) per reaction (data not shown). Standardization of the PCR in this way also rendered the assays highly reproducible: some small differences in fingerprinting patterns were demonstrated repeatedly. The 48 bacterial strains were subjected to PCRs with different primers or primer combinations. Among those were arbitrary primers 933, 961, 963, 1116, 2650, and 2651 (4); primer *mecA1* (12); and the *dru* consensus sequence at the *mec* locus of MRSA DNA, used as two primers (15) (Table 1). The *dru* sequences were synthesized in both the 5′ and 3′ directions. However, neither the primers individually nor combinations of these primers produced satisfying results; either the patterns were identical for most strains or there were no visible DNA banding patterns (data not shown). The identities of finger-

TABLE 2. Comparison of phage typing and PCR fingerprinting of 48 MRSA isolates[a]

Phagovar	Strain no.	Fingerprint with primer or primer pair:			
		935-1026	1204	1014	1014-1026
E1	10	A	I	H	O
	19	A	I	H	P
	20	A	I	H	O
	22	A	I	H	Q
	37	A	I	H	R
	42	A	I	H	Q
	49	A	I	H	S
	50	A	I	H	Q
E2	1	A	I	H	S
	7	A	I	I	O
	11	A	I	H	S
	15	A	I	H	R
	16	A	I	H	Q
	39	A	I	J	S
	40	A	I	H	O
E3	8	A	I	I	O
E2612	2	A	I	H	T
	4	A	I	H	T
	5	A	I	H	T
	12	A	I	H	T
	18	A	I	H	U
	24	A	I	H	U
	32	A	I	H	P
	44	A	I	H	T
	48	A	I	K	T
E2638	28	B	I	K	V
	33	A	II	H	P
	34	A	II	H	P
	43	A	I	H	T
	45	A	I	H	U
E2623	13	A	I	H	Q
	25	A	I	H	P
	29	A	I	I	O
	31	A	I	H	P
E2624	14	C	II	L	P
	26	C	II	L	P
	30	C	II	L	V
	35	C	II	L	V
	41	C	II	L	T
E2627	17	A	I	M	P
E2631	21	D	IV	J	W
E2637	27	A	II	L	P
E2616	6	C	II	L	X
III-49	9	E	III	J	T
	23	F	III	J	T
n	36	G	IV	J	R
	46	G	IV	J	Q
	47	G	IV	J	R

[a] Shown are results for a combined survey of phage typing (13 types, E1 to n) and genotyping (fingerprints A to X and I to IV). The various primers (or combinations thereof) as applied in the PCR tests are indicated. Genotypic characteristics correspond to those shown in the figures. Strains are identified by their laboratory numbers (1 to 50). The total number of strains was 48. The number of variant genotypes (combination of four PCR tests) is 23. Combining DNA and phage typing allows the discrimination of 34 of 48 isolates.

prints obtained with a certain primer or a primer combination imply, alternatively, that the PCR assay itself could be used for confirmation of MRSA. The banding pattern obtained in this way identifies the species as such. Distinct banding patterns of PCR fragments were obtained with primers 935, 1014, 1026, and 1204 either alone or in combination. Subjection of DNA from the 48 different MRSA strains to PCR with primers 935 and 1026 revealed seven different banding patterns (Fig. 1). PCR analysis with primer 1014 revealed six different banding patterns within this group (Fig. 2). When the 48 MRSA strains were subjected to PCR with primers 1014 and 1026, 10 different banding patterns arose (Fig. 3A). PCR with primer 1204 led to the discovery of

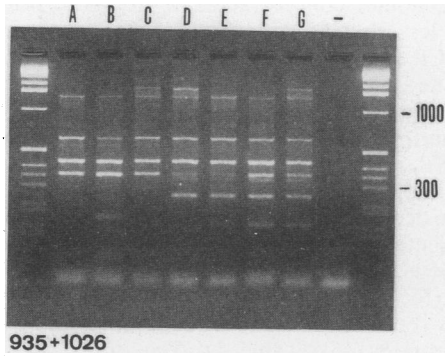

FIG. 1. PCR-mediated DNA typing of MRSA strains. Lanes A to G show the various banding patterns among a collection of 48 MRSA strains. Primers 935 and 1026 were used (see Table 1). −, negative control. The lengths of two of the molecular size markers (1-kb ladder; GIBCO-BRL) are indicated on the right, in base pairs.

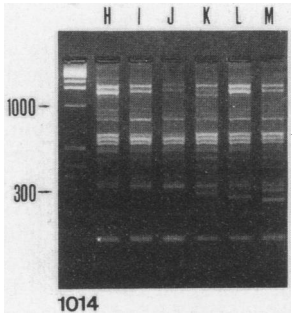

FIG. 2. PCR-mediated DNA typing of MRSA strains. With primer 1014 (see Table 1), six different genotypes, marked H to M, were identified in a group of 48 MRSA strains. The lengths of two of the molecular size markers (1-kb ladder; GIBCO-BRL) are indicated on the left, in base pairs.

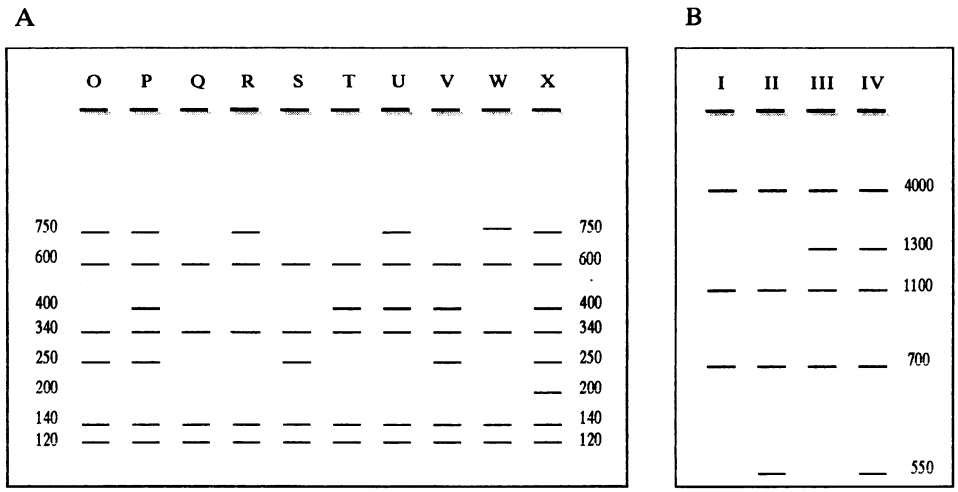

FIG. 3. Schematic presentation of the DNA banding patterns obtained by PCR fingerprinting with a combination of primers 1014 and 1026 (A) and primer 1204 (B) involving DNA from MRSA strains. Displayed are genotypes O to X and I to IV; the lengths of the DNA fragments are indicated. Note that the largest visible DNA fragment visible of type W is slightly longer than similar fragments of types O, P, R, U, and X (A). With primer 1204, longer DNA fragments were synthesized. The lengths of the fragments, as deduced from a comparison with a coelectrophoresed GIBCO-BRL 1-kb length marker, are indicated in the margins, in base pairs.

another four fingerprint types (Fig. 3B). Application of the latter primer resulted in the PCR-mediated synthesis of relatively long DNA fragments. A comparison of the DNA banding patterns seen in Fig. 1 to 3 reveals that a certain size homology exists among DNA fragments found in the various genotypes identified by using these three PCR approaches. PCR with a single primer or a combination of two primers allows the detection of only a limited number of genotypes. Combination of the individual DNA fingerprints obtained with various primers or combinations, however, led to the discrimination of 23 different genotypes in the collection of 48 MRSA strains.

Correlation between DNA fingerprinting and phage typing. The PCR fingerprinting results were compared with results for typing with MRSA-specific bacteriophages (Table 2). Fingerprinting with the combination of primers 935 and 1026 showed a considerable overlap with phage typing. Phagovars E1, E2, and E3 could be grouped as having a single banding pattern (A). Variations of phagovar E could be classified in groups with common characteristics. Some of these groups show the same banding pattern as the former (E2612, E2623, E2627, E2637, and E2638, except strain 28). The other phage type variants (E2624, E2631, and E2616) and phagovar n all displayed unique fingerprints with the combination of primers 935 and 1026.

When primer 1014 was used, several of the aforementioned groups could be differentiated into more subtypes. E2 phagovars, for instance, could be divided into three genotypically different groups (H, I, and J). On the other hand, phagovars E1 and E2624 still appeared to be genetically homogeneous groups. In the E2638 group, one of the isolates (number 28) showed a deviating genotype with either primer 1014 or primers 935 and 1026, which in itself is consistent with nucleotide sequence changes taking place in this particular isolate only. Application of primer 1204 enabled a further discrimination of individual genotypes in the E2638 group. Strains 6, 17, 21, and 27 are single isolates belonging to unique phagovars. The PCR fingerprints for these isolates are, in general, also not found in the other phagovar groups. From Table 2 it can be concluded that, depending on the PCR primers, lysotypically determined groups of strains either can be grouped in a similar way (see primer combination 935–1026 and 1014 for phagovar E1) or can be divided into various genetically different subgroups (see primer combination 1014–1026 for phagovar E2). This implies that, in principle, this PCR approach allows the distinction of the same groups of MRSA isolates, and, in addition to that, it also enables the division of these groups into smaller units. This is of importance in epidemiological analyses, since the higher the discriminative power is, the lower the chances are that independently isolated MRSA strains will still appear to be identical. On the other hand, it should not be forgotten that extremely variable DNA loci are not suited for application in epidemiology, since in this case the resolution of the procedure may be too profound.

Analysis of an MRSA epidemic. The genetic homogeneity of strains isolated during an MRSA epidemic in a geriatric nursing home was studied by the PCR fingerprinting procedure. As a control, the additional MRSA isolate having a unique phage type analysis was included (see Materials and Methods). With primers 935 and 1026 in the PCR genotyping assay, no discrimination could be made between outbreak-related strains from the home for the elderly and the unrelated strain (Fig. 4, upper panel, lanes 5 and 6). All strains displayed genotype F (Fig. 4, upper panel). Primer 1204 again revealed the genetic homogeneity of all outbreak strains. The DNA banding pattern obtained was identical to pattern IV shown in Fig. 3B. Outbreak strains and an unrelated isolate could not be discriminated as well. Interestingly, with primer 1014, outbreak strains and the reference isolate could be discriminated quite clearly. In comparison with genotypes H to M found in the collection of 48 MRSAs, two new PCR fingerprints were obtained. All isolates from the outbreak were genetically homologous, and the independent isolates (Fig. 4, lower panel, lanes 5 and 6) showed a DNA banding pattern which differed from the fingerprints of the isolates from the home for the elderly (Fig. 4, lower panel; the arrow indicates an additional band in the reference strains). In conclusion, the latter observations substantiate the potential applicability of PCR-mediated

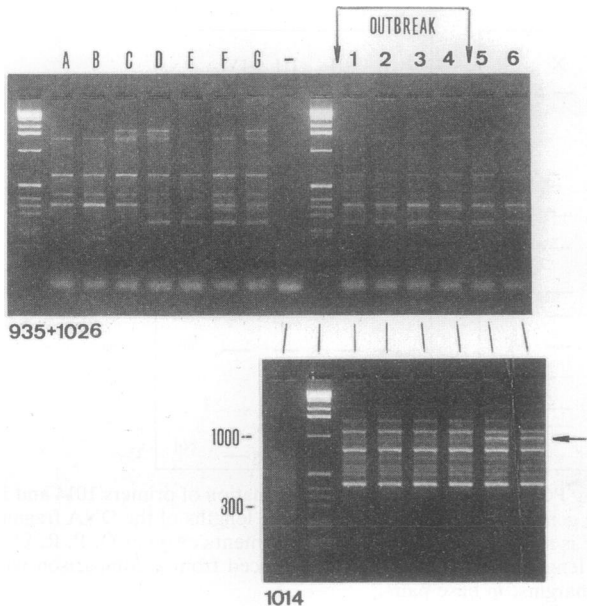

FIG. 4. DNA typing of MRSA strains from diverse sources. Lanes A to G display results similar to those in Fig. 1. Shown is the diversity in DNA fingerprints observed in a group of 48 MRSA isolates. Lanes 1 to 6 contain strains derived either from an MRSA outbreak in a geriatric nursing home (lanes 1 to 4) or from another patient (lanes 5 and 6). The outbreak samples, from which 24 different isolates were available, appear to be genetically homogeneous, as indicated by PCR-mediated genotyping with both primer 1014 and primer combination 935–1026. The latter combination does not enable discrimination of samples in lanes 5 and 6 or 1 to 4. Primer 1014, however, clearly differentiates the large group, which showed identical banding patterns, from the strain isolated from a patient not from the geriatric hospital (see the arrow on the right, indicating an additional band in lanes 5 and 6). The lengths of the molecular size markers (1-kb ladder; GIBCO-BRL) are indicated on the left of the lower panel, in base pairs.

genotyping in epidemiological analyses. Apparently, it is possible to define groups with closely related genetic compositions, which is of utmost importance in comparative analyses of the spreading of certain organisms.

DISCUSSION

Until recently, the method best suited for obtaining direct insight in the variability of genomes was the determination of restriction fragment length polymorphisms. However, this method is complicated and time-consuming and is therefore not very well suited for use in a routine diagnostic setting. The development of PCR methods enabling the identification of complex DNA molecules provides a relatively rapid and simple method to achieve similar goals. Frequently occurring nosocomial epidemics are multiple infections due to the acquisition of MRSA strains. The present method of choice for sorting out these epidemics is typing with MRSA-specific bacteriophages. This procedure is not without pitfalls. However, whereas phage typing may be hampered by phenotypical variation (e.g., growth stage, culture medium, etc.), the PCR typing approach described here is less vulnerable to environmental influences.

On the other hand, the results show that successful application of PCR finger-printing depends heavily on the primer choice. Random amplified polymorphic DNA PCR with short arbitrary primers was not successful. Also, the use of simple sequence-resembling primers or primers based on MRSA *dru* repeats did not enable accurate discrimination of MRSA strains. The best resolution was obtained by applying the enterobacterial repeat consensus primers 1014 and 1204 (Fig. 2, 3B, and 4), a combination of 1204 with an arbitrary primer (1026; Fig. 3A), or the combination of the *mecA2* primer with the same arbitrary primer (Fig. 1). The presence of ERIC-like sequences has not been demonstrated for gram-positive bacteria as yet. It seems that under less stringent PCR conditions, the ERIC1 and -2 sequences encounter some homologies in the genomes of gram-positive bacteria as well. Combining *mecA* sequence-based primers with an arbitrary primer (Fig. 1) illustrates the useful combination of constant methicillin resistance gene sequences and variable DNA sequence motifs. Applying genotyping studies to known and constant regions allows the combined identification of several phenotypic and genotypic characteristics of a given microorganism in one assay. Aiming at both *mecA* sequences and variable DNA loci should allow methicillin resistance determination together with genetic identification of MRSA strains. Several of the primers surprisingly produce fingerprinting schemes that overlap with grouping based on phage typing. Interestingly, several of the bands found in the PCR fingerprints are shared among different genotypes (see, for instance, Fig. 1). Probably this still reflects a close relationship among these isolates, which in the end could serve as an additional means of MRSA identification, and, possibly, strain classification. This also requires examination of methicillin-sensitive *S. aureus* strains. In this study, methicillin resistance of the (clinical) isolates was a prerequisite for inclusion in the study population.

Five phagovars occur only once in the MRSA collection studied. PCR fingerprints of four of these strains are unique as well, which may be considered to support the validity of the PCR method. Twenty-three combinations of DNA banding patterns were identified. When these overall fingerprints were combined again with results of the phage typing, it was possible to distinguish 34 subtypes of *S. aureus* in 48 strains (see also Table 2). It can be imagined that whenever additional primers or primer combinations are used, the discrimination of strains will proceed, resulting in a personal combinatory fingerprint for each strain. The latter is not useful in the examination of epidemics. It was already noticed that concentrating PCR products by ethanol precipitation allows the visualization of additional, minor DNA fragments. However, this severely complicates the analysis and for that reason these results were not included in the figures and tables. It will be interesting to examine the exact nature of the DNA fragments that are synthesized during PCR.

In conclusion, a PCR-mediated procedure which enables straightforward genetic typing of MRSA isolates and, at the same time, correlates well with established phage typing procedures is described. The applicability in the analysis of MRSA outbreaks is demonstrated. Because of the general character of the primers used, this type of PCR-mediated DNA identification procedure can be applied to a multitude of other microorganisms as well. Present studies of non-MRSA isolates, for instance, confirm this latter suggestion (unpublished data). Comparisons between PCR fingerprinting and the accepted molecular techniques applied in epidemiological analyses will have to be made in order to validate PCR genotyping. However, practical application of this PCR-mediated procedure is feasible only when PCR strate-

gies are simplified and when automated systems for reproducible generation and interpretation of DNA fingerprints are available. Finally, elucidation of the way of spreading of microorganisms in clinical settings as traced by PCR fingerprinting can have a significant impact on the measures taken to prevent subsequent infections.

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