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Typing of *Staphylococcus aureus* and *Staphylococcus epidermidis* Strains by PCR Analysis of Inter-IS*256* Spacer Length Polymorphisms

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IS*256* elements are present in multiple copies in the staphylococcal genome, either flanking the transposon Tn*4001* or independent of it. PCR-based analysis of inter-IS*256* spacer polymorphisms was developed for typing of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* strains. Using *Sma*I macrorestriction analysis resolved by pulsed-field gel electrophoresis (PFGE) as the reference method for MRSA typing, excellent reproducibility (100%), discriminatory power (97%), and in vivo stability were observed. Good concordance of the results with those of other molecular typing methods was found for two MRSA collections. Inter-IS*256* PCR analysis of a U.S. collection of MRSA strains ($n = 36$), previously characterized by 15 typing methods, showed more limited discrimination. Agreement was 78% with PFGE analysis and 83% with ribotyping (*Hin*dIII). Analysis of a second set of Belgian MRSA strains ($n = 17$), categorized into two widespread epidemic clones by PFGE analysis, showed 65% agreement. For typing of *S. epidermidis* strains ($n = 26$), inter-IS*256* PCR showed complete typeability (100%) and good discriminatory power (85%). Inter-IS*256* PCR analysis is proposed as an efficient molecular typing assay for epidemiological studies of MRSA or *S. epidermidis* isolates.

Nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) represent an important problem worldwide (5, 31, 40). Typing of MRSA strains is necessary for proper epidemiological investigations of sources and modes of spread of these strains in hospitals and to design appropriate control measures. Traditional typing methods, such as phage typing and determination of antimicrobial-agent resistance profiles, often suffer from insufficient reproducibility, limited discriminatory power, or poor specimen typeability (4, 22, 29, 34). A number of molecular methods have been developed for *S. aureus* typing (11). Restriction fragment length polymorphism (RFLP) analysis techniques, including ribotyping and Southern blot analysis of target sequences present in a single copy, like *mecA* or *agr* genes, reveal a limited diversity among unrelated MRSA strains (4, 11, 34). RFLP probes for mobile elements present in multiple copies in the staphylococcal genome, like insertion sequences (IS*256*, IS*257*, IS*431*, and IS*1181*) and transposons (Tn*554* and Tn*4001*), show greater discrimination than single-copy-gene-based RFLP analysis (2, 12, 19, 22, 34, 42). However, gentamicin-susceptible (Gm^s) *S. aureus* strains are often untypeable by these techniques (12, 22, 34). Among PCR fingerprinting methods, arbitrarily primed PCR (AP-PCR) analysis was found to be epidemiologically useful, but interlaboratory studies showed that there were problems with regard to reproducibility (36). Pulsed-field gel electrophoresis (PFGE) analysis is an accurate and discriminating method which is now used as the reference method for typing *S. aureus* strains in some reference centers (3). However, PFGE analysis is costly and technically demanding, and it still requires interlaboratory standardization (8).

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In this study, we sought to develop a simple, rapid, and accurate PCR-based method for high-throughput typing of MRSA isolates. IS*256* elements were selected as target repetitive elements. These sequences occur in the *S. aureus* and *Staphylococcus epidermidis* genomes either independently or as part of the composite transposon Tn*4001*. This transposon carries the *aacA-aphD* gene, which encodes the bifunctional aminoglycoside-modifying enzyme acetyltransferase AAC(6′)-phosphotransferase APH(2″) (6, 7, 20, 21, 27, 41). We hypothesized that IS*256* insertion positions would be strain specific and spaced close enough to allow amplification of polymorphic inter-IS*256* element sequences. We present the evaluation of this PCR analysis as a novel method for typing MRSA strains. We also show its applicability to typing of *S. epidermidis* strains.

MATERIALS AND METHODS

Bacterial strains. The first group of MRSA strains used in this study (strains 1 to 24 [Table 1]) were selected on the basis of having major distinct *Sma*I macrorestriction patterns differing by four fragments or more (similarity coefficient range, 40 to 80%). These strains were isolated during a multicenter survey from inpatients hospitalized in different Belgian hospitals in 1991 and 1992 (30). The second group of MRSA strains (strains 25 to 54 [Table 2]) were recovered from six persistently colonized and/or infected patients monitored for a period of 2 to 13 months at Erasme Hospital, Brussels, Belgium. The third group of MRSA strains (strains 55 to 90 [Table 3]) were previously characterized by 15 typing methods, including 10 molecular techniques, by Tenover et al. (34) and by two additional molecular techniques by van Belkum et al. (38) and van Leeuwen et al. (39). The fourth group of strains (strains 91 to 107) were selected from among Belgian MRSA strains collected during a multicenter survey conducted in that country in 1995 (31, 33). They belonged to two widespread epidemic clones, as determined by *Sma*I macrorestriction analysis using PFGE as previously described (29): clone 1 ($n = 9$) and clone 2 ($n = 8$). The fifth group of *S. epidermidis* strains (numbers 108 to 133) were selected on the basis of having major distinct *Sma*I macrorestriction patterns, as described above. Moreover, *S. epidermidis* strains analyzed included methicillin-resistant ($n = 11$) and methicillin-susceptible ($n = 16$) strains.

Selection of primers and PCR conditions. The *aacA-aphD* gene in Tn*4001* is flanked by two 1,324-bp-long insertion sequences (IS*256* left [L] and right [R]

TABLE 1. Origin of Belgian MRSA and *S. epidermidis* strains and results of typing by *Sma*I macrorestriction analysis and inter-IS*256* PCR analysis

Strain code	Organism category	City	Hospital	Yr isolated	Susceptibility to:		Type as determined by:					
					Oxacillin	Gentamicin	PFGE[a]	AP-PCR[b]	Coagulase PCR	Protein A PCR	Inter-16S-23S PCR	Inter-IS*256* PCR
1	MRSA	Brussels	I	1992	R	R	1a	bbb	2	4	2	1
2	MRSA	Brugge	II	1992	R	S	2a	ccc	3	3	4	2
3	MRSA	Brussels	III	1992	R	R	3	bab	2	6	2	3
4	MRSA	Brussels	III	1992	R	R	4	bbb	2	4	2	4
5	MRSA	Brussels	III	1992	R	R	5	bbb	2	5	3	5
6	MRSA	Brugge	IV	1992	R	R	6	bbb	2	3	2	5
7	MRSA	Yvoir	V	1992	R	R	7	bbb	2	1	2	6
8	MRSA	Herk-de-Stad	VI	1992	R	R	8	bba	2	4	2	7
9	MRSA	Haine St Paul	VII	1992	R	R	9	ddd	4	4	6	8
10	MRSA	La Louvière	VIII	1992	R	R	10	ddd	4	4	7	8
11	MRSA	Anvers	IX	1992	R	R	11	bbb	2	7	3	9
12	MRSA	Anvers	IX	1992	R	S	12	ddd	5	1	8	10
13	MRSA	Edegem	X	1992	R	R	13	bbb	2	5	9	11
14	MRSA	Edegem	X	1992	R	R	14	bda	2	4	9	12
15	MRSA	Edegem	X	1992	R	S	15	eee	7	3	10	13
16	MRSA	Châtelet	XI	1992	R	S	16	ccc	3	3	5	14
17	MRSA	Châtelet	XI	1992	R	S	17	ffd	6	2	11	15
18	MRSA	Tienen	XII	1992	R	R	18	ddd	5	4	5	16
19	MRSA	Tienen	XII	1992	R	S	19	ffd	6	4	11	15
20	MRSA	Tienen	XII	1992	R	R	20	ddd	5	4	5	16
21	MRSA	Liège	XIII	1992	R	R	21	bbb	2	5	9	17
22	MRSA	Liège	XIII	1992	R	R	22	ddd	4	4	6	8
23	MRSA	Ghent	XIV	1992	R	R	23	bbb	2	4	3	18
24	MRSA	Geel	XV	1992	R	S	24	aba	1	4	1	19
91	MRSA	Brussels	XVI	1995	R	R	1a					1
92	MRSA	Brussels	XVII	1995	R	R	1a					20
93	MRSA	Brussels	XVIII	1995	R	R	1a					21
94	MRSA	Brussels	XIX	1995	R	R	1a					1
95	MRSA	Brussels	XX	1995	R	R	1a					1
96	MRSA	Ath	XXI	1995	R	R	1b					22
97	MRSA	Deurne	XXII	1995	R	R	1b					23
98	MRSA	Edegem	X	1995	R	R	1b					24
99	MRSA	Mechelen	XXIII	1995	R	R	1b					23
100	MRSA	Brussels	XXIV	1995	R	S	2a					2
101	MRSA	Vilvoorde	XXV	1995	R	S	2b					2
102	MRSA	Edegem	X	1995	R	S	2a					2
103	MRSA	Lier	XXVI	1995	R	S	2a					2
104	MRSA	Waregem	XXVII	1995	R	S	2c					2
105	MRSA	Oostende	XXVIII	1995	R	S	2a					2
106	MRSA	Roeselare	XXIX	1995	R	S	2c					2
107	MRSA	Aalst	XXX	1995	R	S	2a					2
108	*S. epidermidis*	Brussels	I	1988	S	R	25					25
109	*S. epidermidis*	Brussels	I	1988	R	R	26					26
110	*S. epidermidis*	Brussels	I	1988	R	R	27					27
111	*S. epidermidis*	Brussels	I	1989	S	R	28					25
112	*S. epidermidis*	Brussels	I	1989	S	S	29					28
113	*S. epidermidis*	Brussels	I	1989	S	R	30					29
114	*S. epidermidis*	Brussels	I	1989	S	S	31					30
115	*S. epidermidis*	Brussels	I	1989	S	S	32					31
116	*S. epidermidis*	Brussels	I	1989	S	S	33					25
117	*S. epidermidis*	Brussels	I	1989	R	R	34					25
118	*S. epidermidis*	Brussels	I	1989	S	S	35					32
119	*S. epidermidis*	Brussels	I	1990	R	R	36					25
120	*S. epidermidis*	Brussels	I	1990	S	R	37					25
121	*S. epidermidis*	Brussels	I	1990	S	S	38					32
122	*S. epidermidis*	Brussels	I	1990	S	R	39					25
123	*S. epidermidis*	Brussels	I	1990	S	R	40					25
124	*S. epidermidis*	Brussels	I	1990	R	R	41					27
125	*S. epidermidis*	Brussels	I	1991	S	S	42					33
126	*S. epidermidis*	Brussels	I	1991	R	R	43					34
127	*S. epidermidis*	Brussels	I	1991	S	S	44					35
128	*S. epidermidis*	Brussels	I	1991	R	S	45					35
129	*S. epidermidis*	Brussels	I	1991	S	S	46					25
130	*S. epidermidis*	Brussels	I	1991	R	R	47					36
131	*S. epidermidis*	Brussels	I	1991	R	R	48					37
132	*S. epidermidis*	Brussels	I	1991	R	R	49					25
133	*S. epidermidis*	Brussels	I	1991	S	S	50					38

[a] The major PFGE types are designated by numerals and include patterns differing by at least four DNA fragments; minor PFGE subtypes are designated by letter suffixes and include patterns differing by at most three DNA fragments.

[b] AP-PCR combined results obtained with Oligo7, ERIC1R and ERIC2 primers.

TABLE 2. In vivo stability of inter-IS*256* PCR patterns in sequential MRSA isolates (n = 30) from six persistently colonized and/or infected patients

Patient	Period (mo)	Anatomic site	No. of isolates	Inter-IS*256* PCR type
P1	5	Wound	7	1
P2	2	Blood	1	1
		Sputum	3	1
		Wound	1	1
		Gastric fluid	1	1
		Blood	1	22[a]
P3	2	Blood	1	2
		Wound	4	2
P4	13	Blood	1	39
		Wound	1	39
		Nose swab	1	39
P5	2	Blood	1	40
		Wound	2	40
P6	11	Wound	4	41
		Nose swab	1	41

[a] Pattern 22 differed from pattern 1 in three fragments.

elements). These IS elements are similar in sequence and occur either in a direct (as in R IS*256*) or an inverted (as in L IS*256*) orientation. They include 26-bp imperfect terminal inverted repeats (IRs) (7). Primers were selected by analysis of the *S. aureus* transposon Tn*4001* DNA sequence (EMBL accession no. M18086) by using the Primer program. Primers P1 and P2 were located within the transposase gene and excluded the IRs (Fig. 1). Significant homologies with other *S. aureus* sequences were avoided. Primers were positioned toward the end of the IS*256* L element and directed outward from this element (Fig. 1). Primer sequences were as follows: P1, 5′-GGACTGTTATATGGCCTTTT-3′ (nucleotides 50 to 30 in the IS*256* L element); and P2, 5′-GAGCCGTTCTTATGGA CCT-3′ (nucleotides 1204 to 1222 in the IS*256* L element).

Genomic DNA was extracted by simple lysis of a single colony as previously described (37). Amplification was performed in 50 μl of reaction buffer containing 5 μl of bacterial lysate, 200 μM each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 0.5 μM each primer, and 1.25 U of AmpliTaq polymerase (Perkin-Elmer Cetus, Emeryville, Calif.) and was carried out in a Gene E thermal cycler (Techne Instruments, Cambridge, United Kingdom). Amplification conditions consisted of an initial denaturation step at 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 45°C for 1 min, and 72°C for 1 min.

Based on the hypothesis that IS*256* or IS*256*-like elements are positioned close enough to allow amplification of intervening sequences, four amplification combinations could occur between two such consecutive elements (in the R or L orientation) when both primers were used. PCR amplification was expected to occur between any two L IS*256* elements, between any two R IS*256* elements, or between an L IS*256* and an R IS*256* element (and vice versa).

Determination of optimal assay conditions for MRSA typing. The annealing temperature was optimized with respect to typeability, discriminatory power, reproducibility, and stability in vivo. Typeability and discriminatory power were assessed by typing a set of unrelated MRSA strains (numbers 1 to 24) that had been classified into distinct clones by PFGE analysis. Since IS*256* elements also occur independently of Tn*4001*, gentamicin-resistant (Gm^r) (n = 17) as well as Gm^s (n = 6) MRSA strains were included. Annealing temperatures of 40, 45, 50, and 55°C were tested.

Discriminatory power was evaluated by calculation of the discrimination index (D) (17) after exclusion of nontypeable strains (32). This index depends on the number of types and on the frequency of distribution of strains of each type. The discrimination index is calculated as follows:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

where N is the number of unrelated strains tested, S is the number of different types, and n_j is the number of strains belonging to the jth type. The discriminatory power was estimated based on results obtained with two sets of strains: 24 Belgian MRSA strains (numbers 1 to 24), previously classified into distinct types by PFGE analysis; and 9 epidemiologically unrelated MRSA strains from the U.S. collection (strains 55 to 63 [Table 3]).

Reproducibility was tested in quadruplicate by comparing patterns obtained by duplicate PCR analysis of duplicate DNA extracts of nine epidemiologically unrelated MRSA strains, each representing a distinct PFGE type, with each run including two Gm^s strains. The index of reproducibility was calculated as recommended previously (32).

In vivo stability. In vivo stability was evaluated by comparison of inter-IS*256* PCR profiles of sequential MRSA isolates recovered over periods of several months from each of several patients (strains 25 to 54).

Comparison of inter-IS*256* PCR analysis with other methods of MRSA typing. Tandem repeat number polymorphisms of the *S. aureus* coagulase and protein A genes were studied by previously described methods (14, 15). To avoid nonspecific amplification, PCR had to be performed with an annealing temperature of 72°C for analysis of protein A gene tandem repeats. AP-PCR analysis was carried out with primers ERIC1R, ERIC2, and Oligo7, used singly as described previously (29, 38). PCR analysis of 16S-23S ribosomal DNA (rDNA) spacer length polymorphisms was done with primers G1 and L1, as previously described (18).

Comparison of independent typing system results is recommended for epidemiological typing (32). If isolates are concordantly grouped into similar types by several typing methods, the probability of isolates being related is increased (32). The concordance of results obtained by inter-IS*256* PCR analysis of three well-characterized MRSA collections (including strains 1 to 24, 55 to 90 [34, 38, 39], and 91 to 107) with those obtained by other typing methods was evaluated. The intermethod concordance was determined as the maximum proportion (percentage) of strains grouped together into unique types by inter-IS*256* PCR analysis and by the other method. On the basis of published results, the performance characteristics of all eight typing methods for MRSA collection 3 were compared (34, 38, 39).

Evaluation of inter-IS*256* PCR analysis for *S. epidermidis* typing. The typeability and discriminatory power of PCR analysis of *S. epidermidis* strains (n = 26) that had been classified into distinct PFGE types were evaluated. These strains included oxacillin-resistant (Oxa^r; n = 10) and Oxa^s (n = 16) strains as well as Gm^r (n = 15) and Gm^s (n = 11) strains (Table 1).

RESULTS

Selection of optimal conditions for inter-IS*256* PCR analysis for MRSA typing. Inter-IS*256* PCR analyses of group 1 MRSA strains showed identical results with annealing temperatures of 50 and 55°C. At these temperatures, Gm^s MRSA strains produced no amplification, resulting in a typeability of only 60%. Only one to nine DNA fragments were produced from Gm^r MRSA strains. By decreasing the annealing temperature to 45 or 40°C, all MRSA strains were typeable by PCR and more informative patterns of amplified DNA fragments were obtained. PCR profiles contained more DNA bands at 40°C (between 4 and 15) than at 45°C (between 4 and 10). All Gm^r MRSA strains displayed a 220-bp DNA fragment which was not observed in Gm^s MRSA strains (Fig. 2).

The reproducibility of PCR testing of duplicate DNA extracts in separate PCR runs was 89% at an annealing temperature of 45°C and 33% at an annealing temperature of 40°C if PCR profiles differing by a single DNA band were considered as distinct. At 45°C, one DNA fragment difference was observed in inter-IS*256* PCR patterns of a single strain, and this difference was found between separate PCR runs (Fig. 2, strain 6). If profiles with two or more band differences were considered as distinct, the reproducibility increased to 100% at an annealing temperature of 45°C and to 67% at an annealing temperature of 40°C (Fig. 2). The discriminatory power was determined to be 98 at 45°C and 99% at an annealing temperature of 40°C if profiles showing two or more band differences were considered as distinct.

Based on these results, inter-IS*256* PCR analysis performed at an annealing temperature of 45°C was selected for MRSA typing. Under these conditions, the assays had 100% typeability, 100% reproducibility, and 98% discriminatory power, based on the interpretation criterion that PCR patterns exhibiting more than one band difference were considered to represent distinct types. A total of 19 types were obtained by PCR, compared to 24 types obtained by PFGE.

In vivo stability. Inter-IS*256* PCR patterns obtained from sequential MRSA isolates suggested that each patient was colonized and/or infected by a single strain. Consecutive isolates from diverse anatomic sites either exhibited identical profiles (29 of 30 isolates) or had profiles that differed by three DNA bands (1 isolate) (Table 2). This suggests that IS elements are

TABLE 3. Comparison of results of molecular typing of a previously characterized U.S. collection of 36 MRSA strains by various methods, categorized by epidemiological relatedness[a]

Category	Strain code	Type as determined by:							
		IS typing[b]	Ribotyping[c]	PFGE[d]	RFLP[e]	Coagulase PCR[f]	AP-PCR[g]	Hybrid-ization[h]	Inter-IS*256* PCR
Unrelated	SA-8	D	e/d	G	I:NH:6:NH[i]	7	CCD	III	42
	SA-12	C	b/b	J	I:A:1:NH	9	AAA	III	43
	SB-18	E1	a/a	A	I:A:1:a	9	AAA	III	44
	SA-11	G	g/d	F	II:NH:6:NH	7	CCD	IV	42
	SA-18	C	b/b	J	I:A:1:NH	9	AAA	V	43
	SA-20	C	b/b	J	I:A:1:NH	9	AAA	VI	43
	SB-1	E	a/a	A1	I:Y:1:a	9	AAA	VII	44
	SB-14	D	e/a	H	I:A:1:NH	9	EDE	III	44
	SB-16	E	a/a	A1	I:Y:1:a	9	AAA	XI	44
Pseudo-outbreaks									
NH-1	SA-1	A	a/a1	K1	I:A:5:a	9	AAA	III	44
	SA-3	C	a/a	A	I:A:1:NH	9	AAA	III	43
	SA-9	A	a/a1	K2	I:A:5:a	9	AAA	III	44
	SA-13	A	a/a	A	I:A:1:a	9	AAA	III	44
	SA-19	A	a/a1	K3	I:A:1:a	9	AAA	III	44
NH-2	SA-5	A	a/a	A	I:A:1:a	9	AAA	III	44
	SA-10	A	a/a	D	I:A:1:a	9	DAA	III	44
	SA-15	A1	a/a	A	I:A:1:a	9	AAA	III	43
	SA-17	A	a/a	A	I:A:1:a	9	AAA	III	44
	SA-2	A1	a/a	A	I:A:1:b	9	AAA	ND[j]	43
Outbreaks									
I	SB-3	E	a/a	A	I:A:1:a	9	AAA	III	44
	SB-5	E	a/a	A	I:A:1:a	9	AAA	III	44
	SB-10	E	a/a	A	I:A:1:a	9	AAA	III	44
	SB-15	E	a/a	A	I:A:1:a	9	AAA	III	44
	SB-19	E	a/a	A	I:A:1:a	9	AAA	III	44
	SB-20	E	a/a	A	I:A:1:a	9	AAA	III	44
	SB-12	E	a/a	A1	I:A:1:a	9	AAA	XI	44
III	SC-1	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-4	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-5	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-9	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-12	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-14	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-17	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-20	F	a/b	A	I:A:4:a	10	BJB	II	44
	SC-11	NH	a/b	A	I:A:4:NH	10	BJB	XII	44
	SC-15	F	b2/b	A	I:A:4:a	10	BJB	III	44

[a] Results of ribotyping, IS typing, RFLP, coagulase PCR, and PFGE analyses are from reference 33; AP-PCR data are from reference 36; hybridization types are from reference 38. Permission to reprint these previously published data has been granted.
[b] RFLP was performed with the IS*431* probe.
[c] Ribotyping results obtained with *Hin*dIII and *Cla*I, respectively.
[d] PFGE types obtained after *Sma*I-digested DNA was separated by either PFGE or field inversion gel electrophoresis.
[e] RFLP types obtained with *Cla*I-digested DNA hybridized with various gene probes: *mec*, Tn*554*, *agr*, and *aph*(2″)-*aac*(6′), respectively.
[f] Coagulase types obtained after PCR and *Alu*I digestion of coagulase gene.
[g] AP-PCR results obtained with three single primers; primers 1, 7, and E2, respectively.
[h] Overall results after hybridization with five strain-specific probes.
[i] No hybridization occurred.
[j] ND, not done.

stable in vivo despite extensive replication of MRSA strains at the various anatomic sites over periods of several months.

Comparison of inter-IS*256* PCR analysis with other PCR-based methods of MRSA typing. Results of four other PCR-based methods for genotyping the group 1 MRSA strains (numbers 1 to 24) are summarized in Table 1. Typeability was 100% for all techniques. The discriminatory power of PCR-mediated RFLP analysis of the coagulase and protein A genes was 71 and 72%, respectively. The combined results of AP-PCR analyses with the three primers provided a discriminatory power of 81%. The best D value (91%) was obtained for 16S-23S rDNA spacer length polymorphism analysis. However, rDNA spacer PCR patterns contained between 4 and 10 DNA fragments with limited variation in size, which ranged between 400 and 700 bp. These patterns were, in some cases, difficult to compare. The reproducibility of PCR analysis of tandem repeat number polymorphisms of the coagulase and protein A genes and for rDNA spacer polymorphism analysis was 100%. It varied, according to the primer used in AP-PCR analysis, from 85% (with ERIC2) to 92% (with Oligo7) (data not shown). Concordance of inter-IS256 PCR typing results with those of other PCR-based typing methods, or the proportion of strains grouped into the same categories by the two methods, was as follows: 33% for PCR-RFLP analysis of the protein A

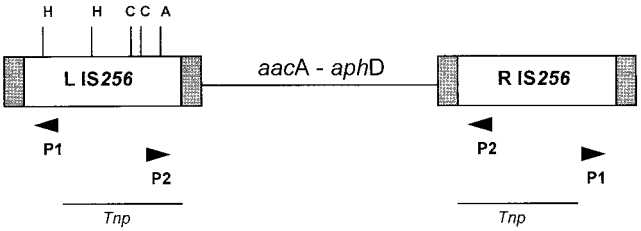

FIG. 1. Structural map of Tn*4001* containing the *aacA-aphD* gene flanked by the left (L) and right (R) IS*256* elements. The locations and directions of primers P1 and P2 are indicated by the arrows. The grey boxes within IS*256* represent the 26-bp IRs. For clarity, only the restriction sites for *Ava*II (A), *Cla*I (C), and *Hae*III (H) are shown. Boldfaced lines below IS*256* R and L represent the location of the potential transposase (*Tnp*) gene.

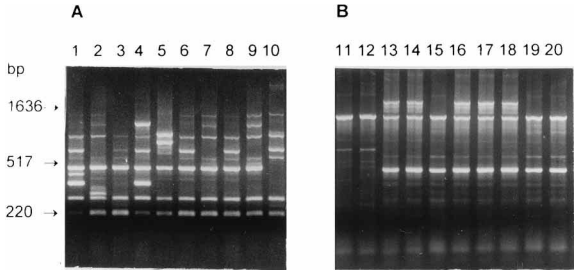

FIG. 3. Representative inter-IS*256* PCR patterns of MRSA strains from Belgium (A) and from the United States (B).

gene, 37% for PCR-RFLP analysis of the coagulase gene, 50% for AP-PCR analysis, and 54% for PCR analysis of the 16S-23S rDNA spacer.

Comparison of inter-IS*256* PCR analysis with other genomic methods of typing MRSA. Results of inter-IS*256* PCR analysis of a previously characterized collection of 36 U.S. MRSA strains were compared with results obtained by seven molecular typing methods (Table 3). Inter-IS*256* PCR revealed a more limited polymorphism in these strains than that observed in Belgian strains. U.S. isolates showed only three PCR patterns, which contained less numerous high-intensity staining fragments and more numerous faintly staining fragments than the Belgian isolates (Fig. 3).

The concordance of inter-IS*256* PCR results with those of other typing methods varied between 42 and 83% (Table 4). The highest intermethod agreement percentages were obtained with ribotyping with *Hin*dIII (83%), RFLP analysis with the *mec* probe (80%), and PFGE analysis (75%) (Table 4). Genomic techniques distinguished between two and seven types (Table 4). The discriminatory power (D) calculated with the small set of epidemiologically unrelated strains in this collection ($n = 9$) ranged from 0 to 83% (Table 4). Inter-IS*256* PCR analysis had a lower D (72%) value than did Southern blot hybridization typing with five probes or PFGE typing (83%) and IS RFLP typing or ribotyping (80%). Although inter-IS*256* PCR analysis showed a limited number of types, similar to that, for example, revealed by coagulase PCR-RFLP analysis, its higher discriminatory power (Table 4) was due to the difference in the number of types in unrelated strains (three versus two) and a more even distribution of unrelated strains into distinct types (Table 3). In the same manner, AP-PCR analysis presented a greater total number of types than did inter-IS*256* PCR analysis (Table 4) but showed a lower discriminatory power due to clustering of many unrelated strains into two types (Table 3). The combined discriminatory power (D) of inter-IS*256* PCR analysis calculated on unrelated Belgian and U.S. strains was 97%.

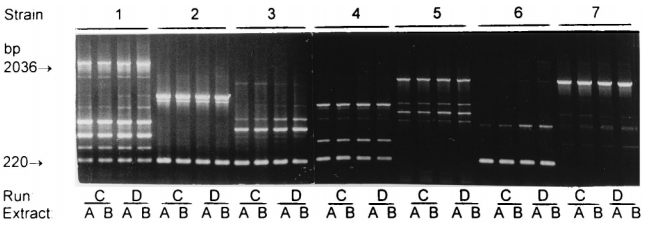

FIG. 2. Reproducibility of inter-IS*256* PCR patterns of MRSA strains (1 to 7), for duplicate DNA extracts (A and B) and in two independent PCR runs (C and D) at an annealing temperature of 45°C. Gm^r strains (strains 1 to 4 and 6) and Gm^s strains (strains 5 and 7) are shown.

Concordance of inter-IS*256* PCR analysis with PFGE typing was also evaluated on the group of epidemiologically related Belgian MRSA strains, which were classified into two epidemic clones by *Sma*I macrorestriction analysis (strains 91 to 107 [Table 1]). Inter-IS*256* PCR analysis allowed recognition and differentiation of these two clones (Fig. 4). Clone 1 strains, which are known to be widely disseminated in all parts of Belgium and have been associated with hospital outbreaks since 1984 (29), showed more heterogeneous inter-IS*256* patterns (displaying up to three fragment differences) than clone 2 isolates, which had indistinguishable patterns (Fig. 4). Clone 2 strains appear to derive from a more limited temporospatial range, as they were first detected in 1992 and were found only in hospitals in the western part of the country. By PFGE analysis, clone 2 strains presented three subclonal variants and

TABLE 4. Comparison of performance of inter-IS*256* PCR analysis with those of other molecular methods for typing a U.S. collection of MRSA strains ($n = 36$)[a]

Typing method	Performance criterion			
	Typeability (%)	No. of types[b]	Discriminatory power (%)[c]	Concordance with inter-IS*256* PCR typing (%)
Ribotyping				
*Hin*dIII	100	4	80	83
*Cla*I	100	3	72	64
RFLP analysis				
IS	97	6	80	42
mec	100	2	22	80
Tn*554*	94	2	48	72
agr	100	4	39	50
aac-aph	78	2	0	75
Coagulase PCR	100	3	39	55
PFGE or FIGE	100	6	83	75
AP-PCR	100	4	55	50
Hybridization	100	7	83	47
Inter-IS*256* PCR	100	3	72	NA[d]

[a] See Table 3 for data.
[b] No. of types found in the complete MRSA collection ($n = 36$).
[c] Values are estimated by the equation D × 100, based on typeable and epidemiologically unrelated strains ($n = 8$ or 9 depending on method [Table 3]).
[d] NA, not applicable.

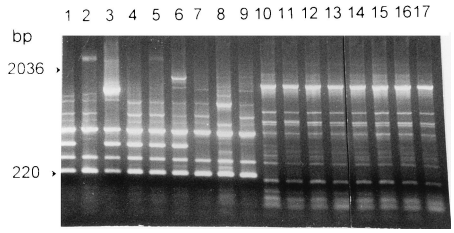

FIG. 4. Inter-IS*256* PCR patterns of MRSA strains classified into epidemic clones 1 (lanes 1 to 9) and 2 (lanes 10 to 17) by PFGE analysis.

clone 1 strains examined here showed two subclonal variants (Table 1).

Use of inter-IS*256* analysis for typing *S. epidermidis* isolates. All *S. epidermidis* strains, irrespective of susceptibility to oxacillin and gentamicin, showed multiple PCR products. A total of 14 PCR patterns differing by at least two DNA bands were observed (Table 1). The PCR patterns had between 1 and 13 DNA fragments, which ranged in size between 134 and 2,036 bp (data not shown). For *S. epidermidis* typing, inter-IS*256* PCR had a typeability of 100% and a discriminatory power of 85%. Interestingly, all Gm^r *S. epidermidis* strains, like *S. aureus* strains, displayed a 220-bp DNA fragment which was not amplified in Gm^s strains.

DISCUSSION

The performance of epidemiological typing systems can be evaluated by using several criteria, including typeability, reproducibility, in vivo stability, discriminatory power, and typing system concordance. In addition, typing should meet convenience criteria, like rapidity, accessibility, flexibility, and ease of use (2, 32). In this study, *Sma*I macrorestriction analysis resolved by PFGE was used as the reference method for typing MRSA strains (29). It is a highly discriminating typing system, with a discriminatory power (D) of between 87 and 100% in previous studies (11). PFGE, however, requires specialized equipment, and it is a rather time-consuming and labor-intensive method. In contrast, PCR-based methods use widely available equipment, are technically simple, and are rapid to perform. Fingerprinting by AP-PCR analysis is limited, however, by problems with reproducibility, due to the low- or very-low-stringency conditions used (36, 38). PCR analyses of single-gene polymorphisms, as applied to *S. aureus*, include PCR-mediated RFLP analyses of the coagulase gene, the X region of protein A, and the *mecA*-associated hypervariable region (11, 14, 15, 24, 28, 34). These methods appear to be highly reproducible but have limited discriminatory power. PCR analysis of interrepetitive element length polymorphisms in *S. aureus* has been described by two groups of investigators; one group analyzed inter-RepMP3 spacer polymorphisms (10), and the second group analyzed polymorphisms of the inter-16S-23S rDNA spacer region (16). These methods of repetitive-element PCR (rep-PCR) analysis were found to be reproducible and moderately discriminating (11). More recently, another rep-PCR typing method, based on analysis of Tn*916*-16S rRNA spacers, was found to provide good discriminatory power (D = 91%) for typing of *S. aureus* strains (9).

The rep-PCR strategy developed in this study relied on the hypothesis that sufficient clustering of IS*256* elements, or sequences partly homologous to IS*256* (called IS*256*-like elements), in the genomes of staphylococcal strains would allow amplification of interrepeat sequences of variable length by using outward primers under moderately stringent conditions. The method of inter-IS*256* PCR analysis reported here allowed typing of all MRSA strains examined, including Gm^s strains that lack Tn*4001*. These findings are in agreement with the results of previous hybridization studies (2, 11, 13, 41) which indicated the presence of isolated IS*256* elements in staphylococcal genomes. In spite of the relatively low-stringency amplification conditions used in this assay, the reproducibility of inter-IS*256* PCR analysis was good, provided that profiles with a single band difference were considered variants of the same type. Amplimer patterns in patients colonized or infected with MRSA were stable over periods of nearly 1 year. These data are in agreement with those of a previous hybridization study that showed the genetic stability of Tn*4001* in multiple MRSA isolates from the same patient (2).

By using *Sma*I macrorestriction analysis as a reference method, excellent discriminatory power (97%) was observed for inter-IS*256* PCR typing of MRSA strains by using the interpretation criteria described above. According to recent guidelines (32), a typing system should achieve a discriminatory index of >95% for reliable assessment of the clonal relatedness of isolates. The discriminatory power of inter-IS*256* PCR analysis compared well with the values reported for other *S. aureus* rep-PCR typing methods (9, 10, 16). In the present comparative evaluation, PCR-based typing methods, such as PCR-RFLP analysis of tandem repeat polymorphisms of the coagulase and protein A genes, exhibited a low level of discrimination, in agreement with previously reported findings, because of clustering of unrelated isolates into some predominant types (14, 15, 34). AP-PCR analysis with multiple primers showed only a limited degree of polymorphism and, moreover, lacked sufficient intralaboratory reproducibility in this evaluation. Interestingly, PCR analysis of rDNA spacer length polymorphisms displayed good discriminatory power. These findings are in contrast with those of Gürtler and Barrie, (16), who found only nine types among 274 MRSA strains, the majority of which originated from two hospitals in Melbourne, Australia. The use of a well-defined collection of bacterial strains for the comparative evaluation of novel typing strategies should be encouraged (32, 34, 36, 39).

Although the sample size was limited, we observed a better power of discrimination with inter-IS*256* PCR analysis for typing MRSA isolates from Belgium than for typing isolates from the United States (discriminatory power, 98% versus 72%). This may be related to a greater diversity of IS*256* insertion sites in European MRSA strains than in American MRSA strains, or it may simply reflect sampling bias. This question requires additional study. A recent study by Morvan et al. (23) showed that European clone 1-related strains, also characterized by having the phage type 77, exhibit IS*256* polymorphisms that can be used to subdivide strains of the same *Sma*I restriction genotype. Despite there being a lower discrimination index with the U.S. collection, inter-IS*256* PCR typing appeared to be superior to the other PCR-based typing methods previously used with this collection.

A good correlation was found between results of inter-IS*256* PCR analysis and those of macrorestriction analysis with the collections of MRSA strains from both Belgium and the United States (34). Isolates showing closely related PFGE profiles, with less than two or three band differences, were interpreted as belonging to the same clonal type (32, 35). Concordance of PFGE clonal types with the results of inter-IS*256* PCR analysis was 78 and 100% for Belgian MRSA strains of clones 1 and 2, respectively. The greater genomic diversity of clone 1 strains can be explained by their wide dissemination, over the last decade, in all parts of Belgium and in neighboring European countries (33). In contrast, clone 2 strains belong to

a more localized and more recently emerged group of epidemic strains that were found in hospitals of western Belgium. A similar phenomenon is observed when PFGE is used, with clone 1 strains showing profiles differing by one to three DNA fragments that were considered as subclonal variants. Guidelines for the interpretation of PFGE patterns to distinguish epidemiologically related and unrelated isolates are now available (32, 35). These interpretation rules relate the variations observed in PFGE patterns to the number of underlying genetic events (32, 35). A similar approach can be considered when interpreting the results of inter-IS*256* PCR analysis. Isolates can be categorized as closely related if their PCR patterns differ by a number of DNA fragments corresponding to a single genetic event (mutation, deletion, or insertion). This single mutational event might lead to the loss of a priming site or might occur between primer binding sites. Further evaluation should clarify whether this type of interpretation criterion is valid for the analysis of inter-IS*256* PCR profiles.

In addition to its use in typing MRSA strains, the method of inter-IS*256* PCR analysis described here appeared to also be effective for the typing of *S. epidermidis* strains. Excellent discrimination was achieved with all strains, including those that were susceptible to oxacillin and gentamicin. The amplification of a well-conserved 220-bp DNA fragment from all Gm^r strains, including those of *S. aureus* and *S. epidermidis*, was interesting. Close duplication of IS*256* or IS*256*-like elements in the *aacA-aphD* resistance gene could explain this phenomenon. Further mapping or sequence analysis should clarify the nature of this DNA fragment.

PCR-based techniques for typing bacterial strains offer the advantages of efficiency and rapidity. Molecular typing methods, like RFLP or PFGE analysis, are accurate but more time-consuming, labor-intensive, and technically complex. The PCR typing technique presented here requires only unpurified DNA template and rapid amplicon analysis by agarose gel electrophoresis. It provides same-day results and fulfills adequately the performance criteria proposed recently by several authors (1, 32). We suggest that the inter-IS*256* PCR assay can be used as a high-throughput screening system for typing methicillin-resistant *S. aureus* and *S. epidermidis* strains, the results of which can be confirmed by other techniques, such as PFGE analysis. In addition, this typing method could be extended to other staphylococcal species or to other bacterial genera, like enterococci, known to harbor multiple copies of IS*256* or IS*256*-like elements in their genomes (25, 26).

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