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Use of an Automated Multiple-Locus, Variable-Number Tandem Repeat-Based Method for Rapid and High-Throughput Genotyping of *Staphylococcus aureus* Isolates

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Fast and reliable genotyping methods that allow real-time epidemiological surveillance would be instrumental to monitoring of the spread of methicillin-resistant *Staphylococcus aureus*. We describe an automated variable-number tandem repeat-based method for the rapid genotyping of *Staphylococcus aureus*. Multiplex PCR amplifications with eight primer pairs that target gene regions with variable numbers of tandem repeats were resolved by microcapillary electrophoresis and automatically assessed by cluster analysis. This genotyping technique was evaluated for its discriminatory power and reproducibility with clinical isolates of various origins, including a panel of control strains previously characterized by several typing methods and collections from either long-term carriers or defined nosocomial outbreaks. All steps of this new procedure were developed to ensure a rapid turnaround time and moderate cost. The results obtained suggest that this rapid approach is a valuable tool for the genotyping of *S. aureus* isolates in real time.

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. The ability to rapidly and reliably identify relatedness between clinical isolates is crucial for the investigation of outbreaks and also for the epidemiological surveillance of strain dissemination. To date, several methods for the genotyping of *S. aureus* isolates have been reported. These methods are based on either phenotypic characterization or molecular techniques (for reviews, see references 38 and 43). Phenotyping usually relies on (i) antimicrobial susceptibility panels, (ii) arrays of specific antibodies directed against bacterial surface components, or (iii) phage susceptibility patterns (38, 48). These techniques may suffer from poor discriminatory power, time consumption, or low throughput (38, 48). Most molecular techniques employ PCR amplification but use different analytical methods for characterization of the amplified DNA fragments. This includes gel electrophoresis for amplicon size estimation, possibly after digestion with a specific restriction enzyme. Sequencing of the amplified fragment often serves as a definitive identification method for amplified DNA fragments. Direct digestion of the intact chromosome followed by size separation (pulsed-field gel electrophoresis [PFGE]) or genotyping by nucleic acid probes (ribotyping) are two techniques that do not require PCR amplification. However, PCR-based techniques appear to be advantageous for the study of slowly growing or difficult-to-grow organisms (26).

PFGE is considered the reference technique for the typing of *S. aureus* strains. However, despite large collaborative efforts (24), its use remains limited by suboptimal reproducibility between laboratories, demanding protocols, low throughput, and high costs (44). Arbitrarily primed PCR methods are less expensive in terms of reagent costs (21), but they also suffer from poor interlaboratory reproducibility. Ribotyping is limited to use with bacterial species that contain several copies of the ribosomal locus (38) and has a lower interstrain discriminatory power than the other techniques (41). Multilocus sequence typing (MLST) shares the advantages of PCR-based methodologies, and the variability of the data obtained by MLST appears to be limited. MLST is currently the "gold standard" method for assessment of the evolutionary relatedness between strains (6, 8); it must be kept in mind, however, that unrelated clinical isolates sometimes display common profiles (38, 48). When *S. aureus* is considered, *spa* typing has been proven to be an inexpensive and rapid typing approach and is based on variable numbers of repeats in the protein A gene (13, 18, 33). Recent sequencing programs have enabled the development of genome-wide microarrays that allow detailed evaluation of the genome contents of the pathogen (10). However, despite its exquisite resolution power, this technology is still too expensive to be used in clinical laboratories.

Each method mentioned above allows the definition of re-

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TABLE 1. Characteristics of oligonucleotides used in the multiplex PCR assay

Target gene	Primer sequence (5′→3′)	Concn (nM)	Amplicon length (bp)		Repeat length (bp)	Source or reference
			Expected in MW2	Measured		
mecA	F-CATTGATCGCAACGTTCAATTT	200				
	R-TGGTCTTTCTGCATTCCTGGA	200	99	99 ± 1	No	This study
sspA	F-ATCMATTTYGCMAAYGATGACCA	400				
	R-TTGTCTGAATTATTGTTATCGCC	400	133	132 ± 2	24	31
spa	F-AGCACCAAAAGAGGAAGACAA	400				
	R-GTTTAACGACATGTACTCCGT	400	284	290 ± 4	9	31
sdrCDE	F-TTACGGATCATGATGATTTCA	300				
	R-CAYTACCTGTTTCTGGTAATGCTT	300	648/554/606	664/580/622	18	This study
clfA	F-GATTCTGACCCAGGTTCAGA	300				
	R-CTGTATCTGGTAATGGTTCTTT	300	1057	945 ± 22	18	31
clfB	F-ATGGTGATTCAGCAGTAAATCC	300				
	R-CATTATTTGGTGGTGTAACTCTT	300	918	880 ± 19	18	31
fnBP	F-GGTCAAGCRCAAGGACCART	200				
	R-AATAATCCGCCGAACAACAT	200	1091	1045 ± 32	40	This study
cna	F-AAAATGACAAAAATGGCAAG	400				
	R-CAGGTTTAGTTGGTGGTGTT	400	1878	1888 ± 125	560	This study

lationships between strains, but direct comparison between respective data sets failed to yield comparable results (20, 22). PFGE is a suitable method for the establishment of clonal relationships, but it has been described to be poorly informative for long-term epidemiological surveillance (4, 5). To the opposite of this, MLST has been validated for long-term and global epidemiological studies (6, 40).

A more recent approach consists of combination of the advantages of different methods with the same panel of strains. For example, two PCR amplifications probing for the presence of toxin genes and the hypervariable region of the *mec* element were applied to a large collection of clinical isolates (25). This technique proved successful for classification of methicillin-resistant *S. aureus* (MRSA) isolates recovered during outbreaks or collected from clinical specimens, while it simultaneously permits evaluation of the potential virulence of these strains (25). Combination of MLST with SCC*mec* type determination also permits the analysis of collections of MRSA isolates and the detection of new MRSA clones (7).

Recently, Sabat and colleagues (31) reported on the use of variable numbers of tandem repeats (VNTRs) for the typing of *S. aureus* using a limited number of target genes. This technique, which is potentially adaptable to different bacterial species (16, 42), appears to offer a good balance in terms of resolution power and cost (42).

We report here on the development of an assay that provides *S. aureus* genotyping together with the assessment of known virulence factors and which facilitates short turnaround times, high sample throughput, and low per-sample costs. We selected a total of eight primer pairs in regions flanking repeat-containing genes that encode bacterial adhesins, a protease, and an immunoglobulin G (IgG)-binding protein. Additionally, a primer pair was selected from the *mecA* gene. Most of these target genes have been previously described as potentially important in the pathogenicity of *S. aureus* (9, 11, 28, 37). A collection of 20 strains extensively characterized by PFGE, MLST, and randomly amplified polymorphic DNA (RAPD) analysis was used for an initial validation of this new procedure (44). The resolution power of this novel automatically analyzed method was then evaluated with a collection of >200 strains from four documented nosocomial outbreaks and 59 pairs of strains collected from long-term carriers. Genotyping results were consistent with PFGE and MLST data for both strain collections.

MATERIALS AND METHODS

Sequence analysis and selection of primers. Repeat-containing target genes from the genomes of reference *S. aureus* strains were selected. A consensus sequence was then identified for each gene by using the BLAST alignment (http://www.ncbi.nlm.nih.gov/BLAST/). After localization of the region of interest, primers (Table 1) were selected by using Jellyfish (version 1.3; Biowire). Particular attention was paid to the amplicon size. All PCR products should have a different size in order to be resolved by electrophoresis.

Strain collections and culture. A panel of 20 clinical MRSA isolates included 10 genetically unrelated isolates (isolates W11 to W20), 5 isolates that exhibited similar but not identical PFGE profiles (isolates W6 to W10), and 5 isolates with undistinguishable PFGE patterns (isolates W1 to W5). A precise description of this strain collection has been published previously (44).

A further set of 212 clinical isolates collected at the Centre Hospitalier Universitaire Vaudois (Lausanne, Switzerland) was composed of (i) 59 pairs of isolates collected from long-term carriers at time intervals ranging from 1 month to several years (Table 2) and (ii) 94 isolates recovered during four distinct nosocomial outbreaks that were collected over 1- to 15-month periods. Isolates collected during each of these four outbreaks displayed nondistinguishable SmaI digestion profiles by PFGE (Table 3). All strains were plated on Mueller-Hinton agar, and a single colony was used for the lysis step (see below). All isolates were compared with three reference strains whose genomes have been sequenced previously: *S. aureus* N315, Mu50, and MW2 (2, 19).

Optimization of bacterial lysis. Single colonies picked from blood agar plates (strains N315 and MW2) were suspended in 200 μl Tris-EDTA buffer (TE; 10

TABLE 2. Sequential isolates from 59 MRSA carriers sampled at time intervals of 31 to 800 days

Patient no.	PFGE						VNTR analysis	
	Isolate 1 strain no.	Isolate 1 SmaI pattern	Isolate 2 strain no.	Isolate 2 SmaI pattern	No. of bands different between isolates 1 and 2	Interval between samplings (days)	Distance (%)[a]	Cluster type(s) for isolates 1 and 2[b]
1	3	A	4	A	0	767	0.9187	A-A
2	5	A	6	A	0	432	1.2847	A-A
3	7	A	8	A	0	330	1.9567	A-A
4	13	A	14	A	0	44	9.1058[c]	A2-A3
5	21	A	22	A	0	101	0.637	A-A
6	49	A	50	A	0	347	3.5113	A-A
7	81	A	82	A	0	63	0.5367	A-A
8	83	A	84	A	0	82	2.8817	A-A
9	85	A	86	A	0	539	1.1944	A-A
10	89	A	90	A	0	134	0.6931	A-A
11	93	A	94	A	0	68	0.3602	A-A
12	95	A	96	A	0	54	28.2947[c]	A1-A6
13	97	A	98	Aj	6	214	1.7809	A-A
14	99	A	100	A	0	96	0.9844	A-A
15	101	A	102	A	0	108	0.9919	A-A
16	111	A	112	An	2	124	2.1468	A-A
17	115	A	116	A	0	169	0.5399	A-A
18	117	A	118	A	0	31	17.0523[c]	A1-A5
19	121	A	122	A	0	43	0.5024	A-A
20	125	A	126	A	0	104	1.2395	A-A
21	133	A	134	A	0	55	42.5202[c]	B-A
22	135	Ac	136	Ac	0	50	0.4995	A1-A1
23	9	C	10	C	0	285	0.5801	B-B
24	15	C	16	C	0	83	4.4952	B-B
25	17	C	18	Cb	2	184	3.5772	B-B
26	19	C	20	C	0	33	0.5868	B-B
27	39	C	40	C	0	42	0.2429	B-B
28	67	C	68	C	0	77	0.4621	B-B
29	139	C	140	C	0	42	0.6546	B-B
30	23	4h	24	4h	0	41	0.3421	C-C
31	25	4h	26	4h	0	118	2.7194	C-C
32	27	4h	28	4h	0	107	1.2657	C-C
33	29	4h	30	4h	0	38	0.9165	C-C
34	33	4h	34	4h	0	100	0.5886	C-C
35	35	4h	36	4h	0	100	14.9817[c]	C-C1
36	61	10	62	10	0	94	0.9419	B1-B1
37	63	10	64	10	0	41	1.0679	B1-B1
38	75	10	76	10	0	77	0.3062	B-B
39	77	10	78	10	0	185	0.165	B1-B1
40	91	10	92	10	0	34	0.4918	B-B
41	105	10	106	10	0	371	0.4987	B1-B1
42	73	10e	74	10e	0	83	0.6818	B-B
43	131	11	132	11	0	47	0.2004	B1-B1
44	113	13	114	13	0	70	0.2517	B-B
45	137	13	138	13	0	229	0.3546	B-B
46	87	18	88	18a	1	800	1.2829	B-B
47	79	19	80	19	0	37	0.2235	C1-C1
48	127	33	128	33	0	222	0.4565	C-C
49	123	37	124	37	0	34	0.3595	C1-C1
50	107	17e	108	17e	0	114	6.1733	B-B
51	71	17f	72	17g	4	80	1.1087	C1-C1
52	45	55a	46	55a	0	60	1.8613	C-C
53	129	5a	130	5a	0	55	0.5868	D-D
54	109	5f	110	5	2	287	0.4625	D-D
55	69	7a	70	7a	0	153	8.5125*	B-B1
56	65	7g	66	7g	0	57	2.0724	B-B
57	31	Da	32	Da	0	45	1.5353	C-C
58	103	5	104	5b	2	517	0.6063	D-D
59	119	8	120	8	0	198	6.2747	B-B

[a] Distance calculated between the pair of strains considered.

[b] Clusters were denominated A (the larger one) to D, and subclusters were defined by using the 7.5% cutoff value (see Results). Strains showing distances ranging from 0 to 7.5% were considered to belong to the same cluster. An additional 7.5% distance value defined subclusters (indicated by numbers) containing related but not clonal strains.

[c] Pairs of strains showing a significant distance difference.

TABLE 3. Global characteristics of four nosocomial outbreaks caused by strains showing distinct PFGE profiles

Episode no.	Strain no.	SmaI profile	No. of strains	Duration (mo)
1	141-147	7	7	1
2	148-166	B	19	2.5
3	167-203	A[a]	37	12
3	204–212		44	
4	213-243	A[a]	31	15
4	244–245		33	

[a] Different profiles were detected.

mM Tris and 1 mM EDTA, pH 8) and then mixed with 100 mg glass beads (diameter, 100 μm; Schieritz and Hauenstein, Switzerland). The cells were disrupted for increasing periods of time (10 to 180 s) by using a MixerMill (QIAGEN) at maximum frequency (30 Hz). After centrifugation at 5,000 rpm for 2 min, the supernatant (approximately 140 μl) was directly used in the PCR amplification assay with primers specific for *clfB* (predicted amplicon size, 918 bp in MW2) or *cna*, the largest predicted amplicon (1.8 kb in MW2). The PCR products were purified by using QiaQuick columns (QIAGEN) and eluted in a volume of 10 μl of pure water. Purified material was immediately analyzed by using the BioAnalyzer on a DNA 12,000 chip (Agilent, Palo Alto, CA). This procedure was compared to a previously described DNA extraction method involving lysostaphin treatment (12).

PCR amplification, separation of amplicons, and reproducibility. Nucleic acids from the three reference strains were simultaneously assayed with the 20 clinical isolates, which were used as positive controls (data not shown). Conditions for amplification were as follows: first step (t_1), 2 min at 95°C; t_2, 15 s at 95°C; t_3, 20 s at 60°C; t_4, 40 s at 72°C (t_2 to t_4, repeated 35 times); and t_5, 10 min at 74°C. The total volume of each PCR mixture (KOD Hot Start; Novagen) was 20 μl and contained 0.8 U of *KOD* polymerase and all primers listed in Table 1 at the indicated concentrations. After PCR amplification, 1 μl of each PCR mixture was loaded on a DNA 12,000 chip and run on the BioAnalyzer. Agilent 2100 instrument. Expert application was used to monitor the overall quality of the experiments.

The variability of the PCR and the amplicon migration time were evaluated by using DNA from MW2, a strain that amplified the eight targets. The PCR was performed in triplicate with four independent DNA extraction products (12 samples). Each PCR amplification product was randomly loaded four times on four BioAnalyzer chips.

The reproducibility of the whole procedure was also estimated by using Witte's strain collection (44, 47). The complete protocol was performed three times with independent samples. This experiment was used to define the cutoff values that could be used to distinguish related from unrelated strains.

Data analysis. BioAnalyzer output files containing raw fluorescence data were exported into specifically developed software. The fluorescence data were rescaled according to their ladder positions to allow comparison of the results for multiple samples. The relative distance between the rescaled data was assessed by cross-correlation coefficient calculation (1), which consists of comparison of the fluorescence values between samples at each time point. The results were subjected to hierarchical clustering by the unweighted pair group method with arithmetic means (UPGMA) (30). START (17) was used to produce trees with the RAPD analysis or MLST data (44, 47).

Southern blotting. DNA manipulations were performed by standard methods (32). Genomic DNA from *S. aureus* was prepared by the method of Muller et al. (23) and digested with HindIII (New England Biolabs), as directed by the manufacturer. Probes specific for *S. aureus spa* and *sdr* (Table 1) were constructed by PCR amplification from genomic DNA of strains W2 and W7, respectively, in the presence of digoxigenin-coupled dUTP (Roche Diagnostics). Southern hybridizations were then performed with strains (i) W2 and W5 with the *spa* probe and (ii) W7 and W8 with the *sdr* probe (36).

RESULTS

Reagents and labor costs. The fixed cost of the BioAnalyzer 2100 apparatus is $19,500. The DNA chip kit (300 samples) costs $331. PCR primers cost approximately $80 and allow a few thousand reactions. The enzymatic kit for 1,250 reactions (20 μl) costs $1,100. These prices are based on those in the manufacturers' catalogues. Variable costs were calculated as approximately $2.00 to $2.50 per determination, including pipette tips. The labor time for analysis of 12 strains mainly consists of the preparation of bacterial suspensions and the performance of bacterial lysis (15 min). The preparation and cycling procedure requires about 100 min. Microcapillary electrophoresis lasts 35 min; file export and automated analysis are immediate. Thus, the whole procedure requires approximately 3 h, with about 40 min of hands-on time.

Development and automation of the typing assay. Figure 1A shows that there is a linear correlation between the bacterial lysis time and the amounts of total DNA extracted. The maximal amount of DNA was recovered from samples subjected to 180 s of lysis. The quality of the extracted DNA was then evaluated by using two primer pairs that amplify a 918-bp fragment of the *clfB* gene (Fig. 1B) or primer pairs that amplify a 1,878-bp fragment of the *cna* gene (Fig. 1C). A minimum of 90 s of lysis was required to provide high fluorescence signals for both gene targets. However, whereas 180 s yielded similar signal intensities for the shorter PCR product, the larger fragment showed a marked decrease in fluorescence intensities after 90 s of lysis. The average size of the DNA smears obtained varied from 11.5 kb for the shortest lysis time to approximately 5.6 kb for the longest lysis time. Thus, the decreased yields of the *cna* amplicons are likely due to the DNA shearing generated by prolonged lysis. The optimal bacterial lysis duration was estimated to be 90 s, as confirmed by the intensities of the fluorescence signals obtained after lysostaphin treatment (data not shown).

Table 1 indicates all primer sequences and their respective concentrations used in the multiplex reaction following optimization on purified or crude DNA extracts as the template. The expected sizes of the amplicons (determined on the basis of the strain MW2 genome sequence) and the measured sizes are also indicated and showed an excellent concordance. All primer pairs were first tested in a simplex PCR to assess the length of each amplification product (Table 1). Additional optimization of the primer concentrations was performed to improve the robustness and the reproducibility of the assay and to obtain comparable amounts of all amplification products. Strain MW2 (which contains all eight target genes) and strain N315 (which contains all but the *cna* gene) were used to optimize the whole procedure (data not shown).

Figure 2 shows representative BioAnalyzer profiles determined with these two strains. The profiles revealed differences in the amplicon sizes between the two strains and the presence of an additional band in strain MW2 (band 8; collagen adhesin [*cna*]), as expected from genome sequence information.

Reproducibility of the PCR amplification and analysis properties. The size resolution of the BioAnalyzer is not linear over all target sizes but can be decomposed in three regions with different migration behaviors: (i) targets between 0.05 and 1 kbp are resolved at 50 s/kbp, (ii) targets between 1 and 5 kbp are resolved at 5 s/kbp, and (iii) targets ranging from 5 to 12 kb are resolved at 0.8 s/kbp. The last region displays the lowest resolution capacity and was not used in our assay. The reproducibility of the whole procedure was assessed by using strain

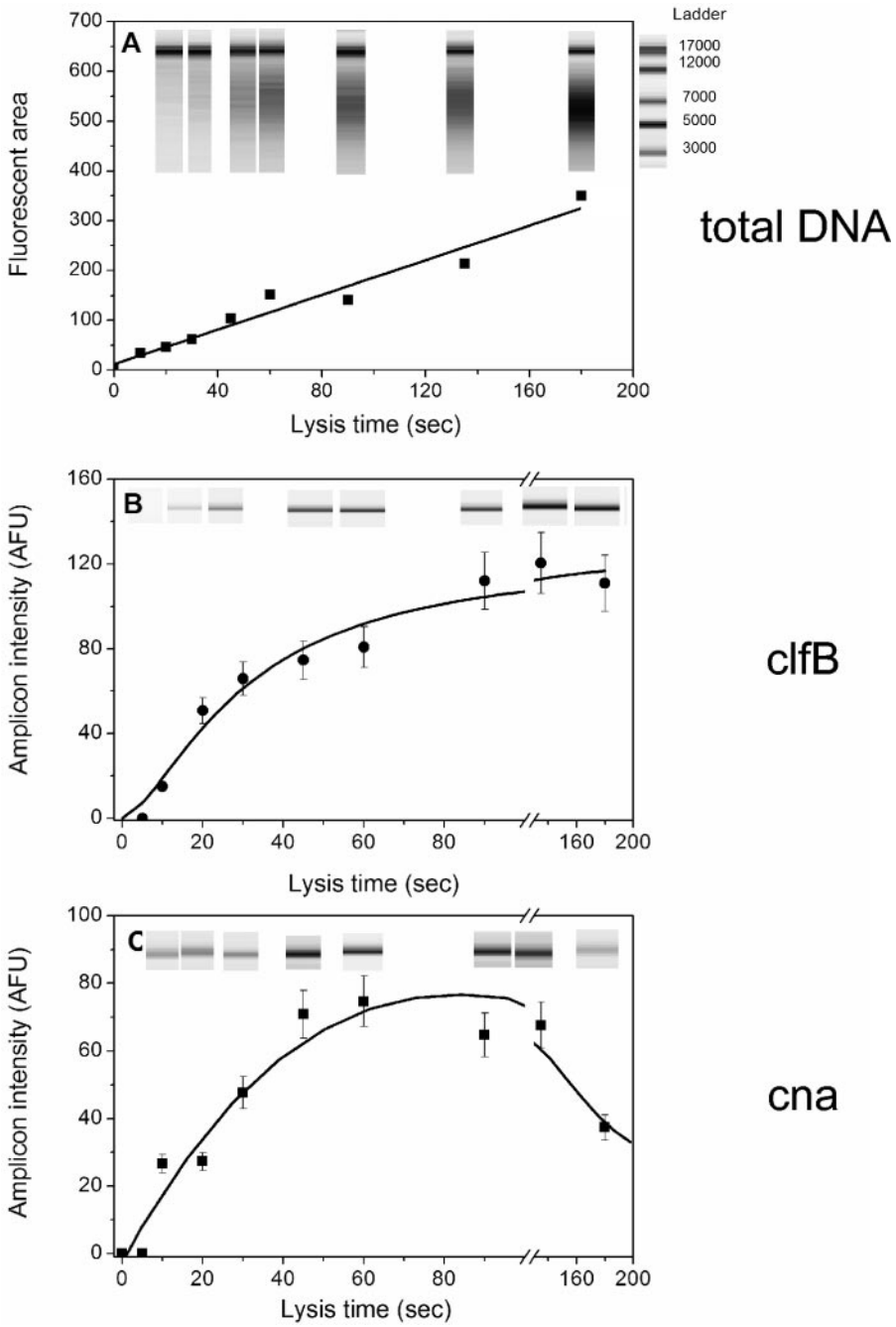

FIG. 1. Effect of lysis duration on DNA yields and PCR amplification results. (A) Amounts of total purified DNA obtained after the rapid lysis procedure (strain MW2), as determined by the fluorescence area under the curve (BioAnalyzer). The electropherogram shown at the top indicates the corresponding lysis time points. (B) Fluorescence intensities of a middle-size *clfB* amplicon (approximately 0.9 kb) as a function of the lysis time. Calculated DNA amounts and the electropherograms at the top show dose dependence, with maximal values between 90 and 120 s (C). Fluorescence intensities of the large *cna* amplicon (1.8 kb) as a function of the lysis time. Calculated DNA amounts and the electropherograms at the top show increasing yields until 90 s, followed by a major decrease for longer lysis times. The numbers next to the electropherograms are in base pairs.

MW2, which generates the eight target amplicons. All amplification products were observed in the 48 determinations. The only parameter that varied was the migration times of the amplicons, which showed a maximal variation of ±0.25 s for all amplicons.

Triplicate experiments performed with the Witte collection yielded comparable clustering trees (data not shown). Each individual strain showed a distance variation that ranged from 0 to 7.5%. Thus, we considered a minimal distance of 7.5% as the cutoff value for cluster formation. An additional 7.5% distance value defined subclusters (indicated by numbers) containing related but not clonal strains.

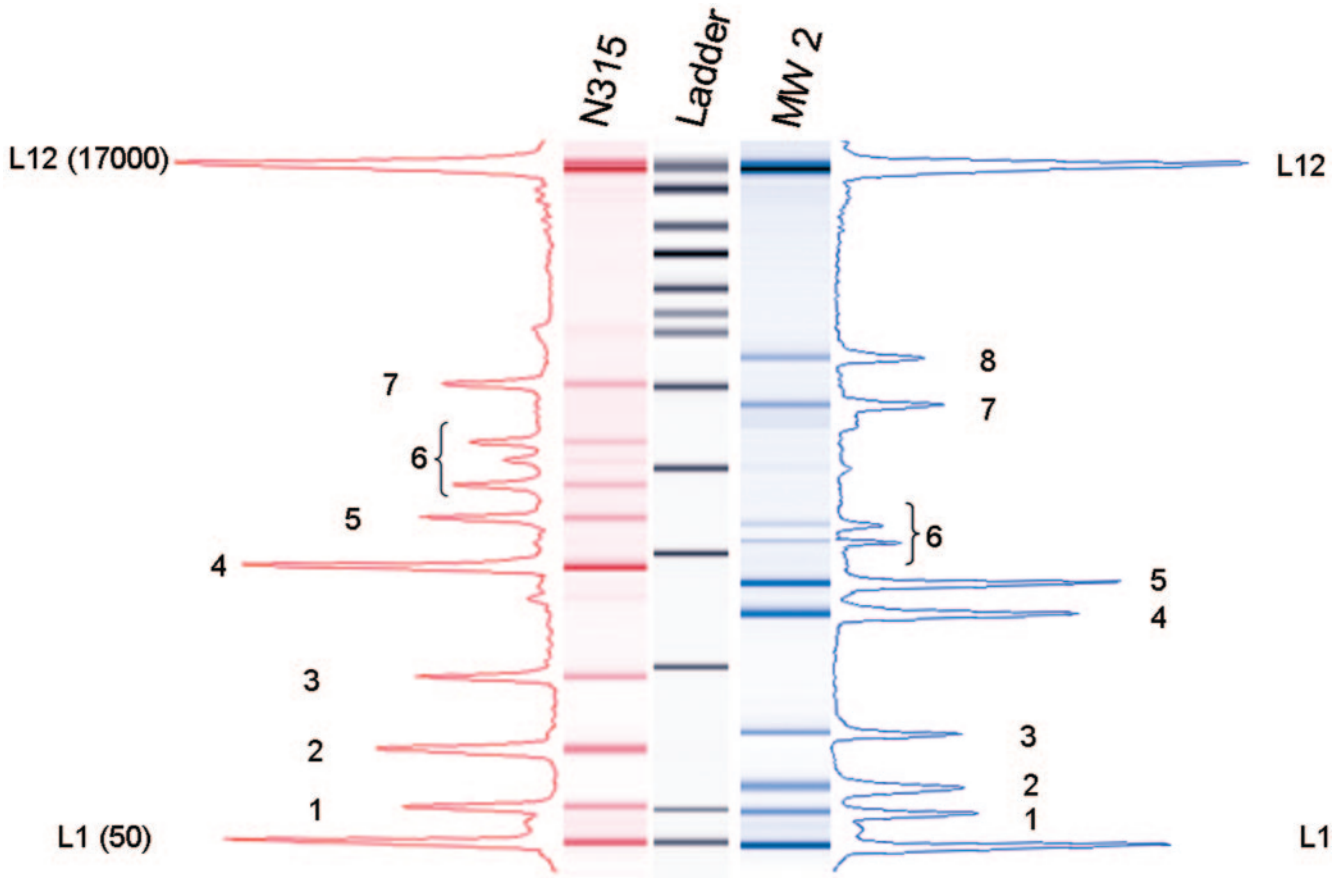

FIG. 2. Comparison of electropherograms obtained for the strains sequenced. Representative electropherograms and the corresponding gel patterns (BioAnalyzer) obtained for strains MW2 and N315 are shown. Strain MW2 displays eight peaks, while strain N315 shows only seven peaks. Peak numbers correspond to 1, methicillin resistance gene (*mecA*); 2, V8 serine protease (*sspA*); 3, protein A (*spa*); 4, Ser-Asp-rich fibrinogen-binding protein (*sdrCDE*); 5, clumping factor B (*clfB*); 6, clumping factor A (*clfA*); 7, fibronectin-binding protein (*fnBP*); and 8, collagen adhesin A (*cna*), as determined by simplex PCRs with each individual primer pairs.

Comparison of the new procedure with established typing methods. A collection of 20 strains extensively characterized by PFGE, MLST, and binary typing (44) was used for initial validation of this new procedure. The clustering tree obtained by VNTR analysis (Fig. 3A) was compared with those previously obtained by MLST (Fig. 3B) and binary typing (Fig. 3C) analyses. The three methods, MLST, binary typing, and our assay, showed a cluster containing strains W1 to W5. The MLST and VNTR trees revealed that W17 was the closest neighbor of the cluster containing strains W1 to W5. The other major cluster, composed of closely related strains W6 to W10, was also present, according to the results of both techniques. However, as opposed to all other methods used to characterize this collection, MLST found that strains W13 and W16 were clonal. Other isolates (W12, W15, and W20) appeared to be unrelated by using binary typing or RAPD analysis. Based on these results, the calculated discriminatory indices (15) were 94, 91, and 90 for the VNTR, MLST, and binary typing approaches, respectively.

Two differences were observed among clusters containing related or clonal strains according to the results of PFGE, MLST, or RAPD analysis. Strain W5 showed an altered *spa* signal amplicon compared to those for strains W1 to W4. Whereas this signal consisted of a single 300-bp band in strains W1 to W4, it led to triplet bands in strain W5, with apparent molecular sizes of 300, 330, and 360 bp, respectively (Fig. 4A). This difference was not related to different gene copy numbers, since strains W1 to W4 and W5 showed similar Southern blot patterns (Fig. 4B). Thus, this difference probably reflects multiple hybridization sites for one primer on the target gene in strain W5. Another difference was observed in the cluster containing strains W6 to W10. Strain W8 was shown to lack the *sdr* gene signal and displayed different patterns (Fig. 4C). Southern blot hybridization revealed a different pattern (Fig. 4D) that reflected sequence diversity in the *sdr* locus between strain W8 and the other members of this cluster.

To evaluate the stability of the targets used in the VNTR reaction over time and to assess the discriminatory power of the assay, we tested two collections of clinical isolates containing >200 strains. A collection of 118 clinical isolates consisting of 59 pairs of strains sampled from the same patients across time intervals ranging from 31 to 800 days was first analyzed. By PFGE and repetitive extragenic palindromic PCR, the 59 pairs of strains were considered clonal. This collection contained 28 clonotypes (Table 2). The main clusters contained 22, 7, 7, and 6 pairs of strains of types A, C, 10, and 4H,

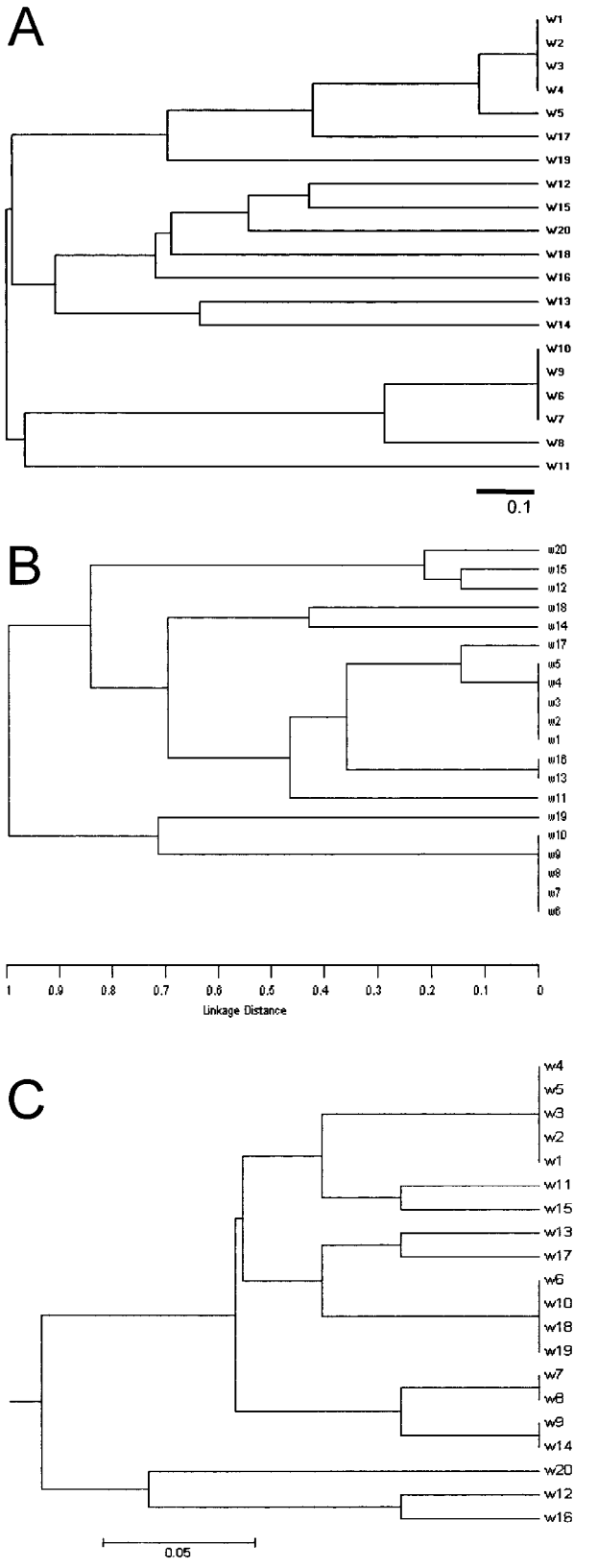

FIG. 3. Clustering trees. The trees were obtained by the new VNTR method (A), MLST (B), or RAPD analysis (C) by clustering of the data by the UPGMA method. The tree generated by the new method (A) presents more similarity to that obtained by MLST.

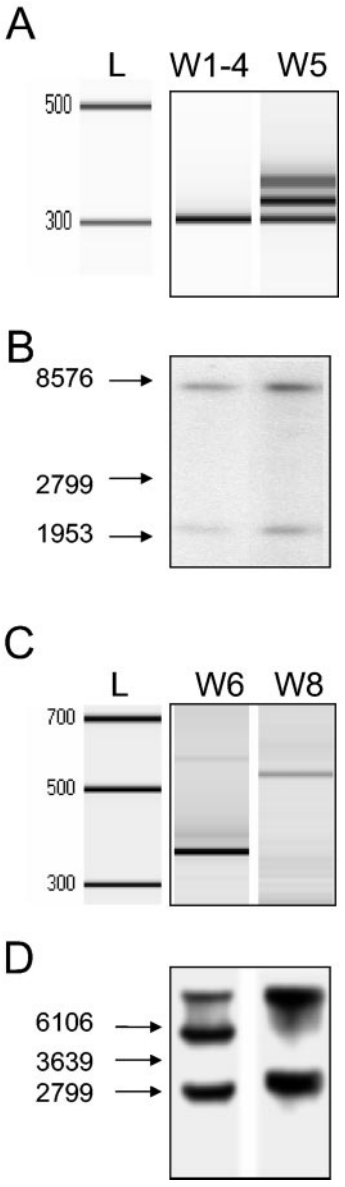

FIG. 4. Analysis of subclonal differences. Magnification of the region showing differences between isolates considered clonal by MLST. Differences involved the *spa* gene (A) for strain W5 and the *sdr* locus for strain W8 (C). Southern blots were performed with the genomic DNA of strain W5 (B) or with the genomic DNA of strain W8 (D) with a digoxigenin-labeled probe.

respectively, and 10 unique clonotype pairs, as previously determined by PFGE (5). Among these 59 pairs of strains considered clonal by PFGE, 53 pairs were also found to be clonal by our VNTR approach, while 6 pairs appeared to be different, as shown in Table 2. For five pairs of strains, both isolates appeared in related clusters, and the isolates in one pair showed totally different profiles. For pairs of strains showing similar SmaI PFGE profiles, major clusters were correctly resolved by the new assay, confirming the stability of the profiles over long periods of time. Other minor pulsotypes were distributed in these main clusters, suggesting genetic relatedness between apparently different PFGE types (Table 2). For ex-

ample, in our VNTR assay, PFGE types 4H and 10 appeared close to the type A and C SmaI profiles, respectively.

A collection of 94 strains derived from four separate nosocomial outbreaks was also tested by our assay. The general profiles of the VNTR clustering tree (Fig. 5) were concordant with the PFGE profiles (Table 3) and also segregated the 94 strains into three main clusters. Indeed, the third and fourth epidemic episodes showed a similar SmaI PFGE type (type A). The VNTR clustering tree revealed a scattered distribution of strains arising from these two episodes, all within the same cluster. These two outbreaks happened in different hospitals in the same geographical area and showed a maximal strain divergence of 20%, according to VNTR. Finally, for the third and the fourth episodes, different SmaI PFGE profiles were recorded for subsets of seven and two strains, respectively. These strains were also segregated into distinct clusters by VNTR, as shown in Fig. 5.

DISCUSSION

We report on a rapid VNTR-based approach that allows the genotyping of large numbers of *Staphylococcus aureus* isolates per day. When it is considered that 12 strains can be analyzed simultaneously by use of the BioAnalyzer, the whole procedure takes only 2.5 h to deliver results. With the well-documented Witte collection (44, 47), the new procedure showed a resolution power equivalent to those of reference methods, such as PFGE and MLST, for resolution of clusters of clonal strains. For related strains W6 to W10, this new assay and MLST showed comparable profiles, as opposed to binary typing, confirming the robustness of this method for outbreak analysis (45, 46). Interestingly, one cluster of five strains (W1 to W5) that were previously not distinguishable by PFGE or binary typing could be differentiated by this rapid molecular assay. Another cluster composed of related strains (W6 to W10) that were previously not distinguishable by RAPD or binary typing and that differed by only one band by PFGE also revealed different profiles by this new method. Thus, the detection of two unexpected molecular differences suggests that this novel assay may provide high typing resolution.

We have shown that the VNTR-based procedure provides adequate resolution power for long-term typing, proving the stability of the selected target genes. This technique was also shown to be applicable to the discrimination of clinical isolates responsible for epidemic episodes. This property was recently suggested by using the variability of a single target (*spa* typing) that yielded results congruent with those obtained with a whole-genome DNA microarray (34). In this study, genetic diversity was estimated by a large variety of typing techniques and showed that *spa* typing was the best method after analysis with DNA microarrays, a technique that measures thousands of parameters.

Based on the results obtained with our VNTR approach and the robustness of the analysis software, the use of several repeated regions yielded a rapid and powerful assay that was able to determine clonality and relatedness in either epidemic or endemic settings. This property is likely due to the selection of short-term and long-term markers among the genes targeted in the multiplex reaction. These targets conferred a high resolution power, as reported for other bacterial species (14, 29, 39); reliability for the analysis of strains from outbreaks (14); and also reliability for examination of the phylogeny of geographically and temporally separated strains (35). Our results and those obtained by others suggest that the evolutionary

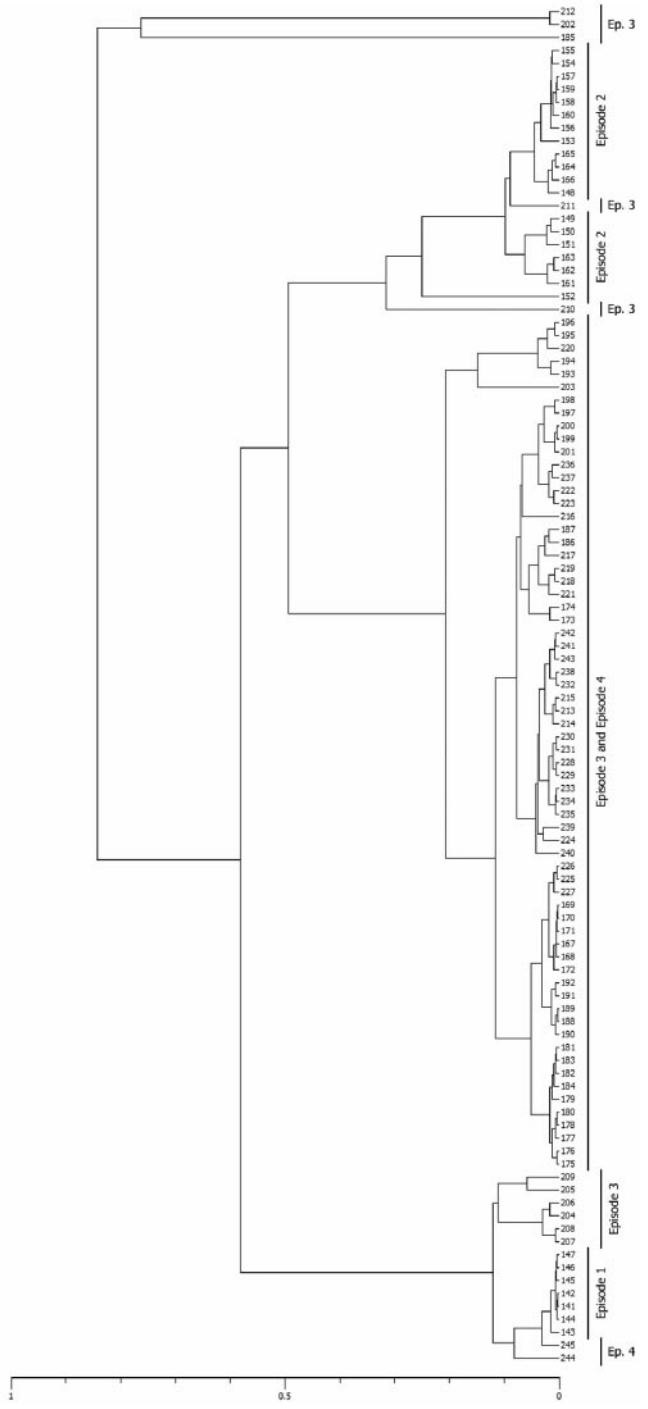

FIG. 5. Analysis of collections of clinical isolates from epidemic episodes (Ep.). VNTR analysis of four outbreaks showed three different clusters, plus one group of two related clonotypes. Groups of strains for each cluster based on their SmaI PFGE profiles are indicated on the right. The scale indicates the level of genetic relatedness within this set of strains based on UPGMA analysis.

frequency of VNTR-containing genes is also adapted to the study of transmission events. The discriminatory power of our eight target-based VNTR approach appears to be appreciable, as groups of strains sharing identical SmaI PFGE profiles appeared to be only related and not clonal, and they remained segregated in subdivisions of large clusters.

The simplicity of this method, which relies on a single multiplex PCR amplification and a totally automated clustering analysis, limits the risk of user-dependent variation, such as that encountered in image analysis-based techniques (3, 26). As suggested recently (27), PCR-based techniques dedicated to genotyping are powerful and allow identifications to be made rapidly, even when slowly growing bacteria are studied. PCR is widely used in clinical laboratories, and the intercenter variability of this technique is probably not a major concern (34). These characteristics, coupled with the possibility of typing dozens of strains every day, should enable large-scale prospective molecular typing for outbreak analysis in real time, an important feature for the evaluation of nosocomial MRSA transmission and control (34). The moderate reagent costs per sample of the method and its overall performance suggest that this approach represents a valuable tool for the genotyping of *S. aureus* isolates in less than 3 h. This assay still warrants multicenter evaluation to assess the possibility of the exchange of genotypic data for the epidemiological monitoring of geographically unrelated patients.

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