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Comparison of PCR-Based Methods for Typing *Staphylococcus aureus* Isolates

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In this study, we compared the potentials of (i) a multiplex PCR-based multilocus variable-number tandem repeat (VNTR) assay; (ii) a triplex PCR coamplifying fragments of *spa*, *coa*, and the hypervariable region adjacent to the *mecA* gene; (iii) restriction profile analysis of the STAR repetitive element; (iv) randomly amplified polymorphic DNA analysis; (v) inter-IS*256* PCR; and (vi) rep-MP3 PCR. Multilocus VNTR typing and triplex PCR (*coa*, *spa*, and hypervariable region) approaches showed excellent reproducibility and high discriminatory power; however, only multilocus VNTR typing could distinguish all pulsed-field gel electrophoresis and *spa* types. Multilocus VNTR typing appears to be the most useful PCR-based method for the rapid genotyping of *Staphylococcus aureus* strains.

Staphylococcus aureus is an increasingly common cause of nosocomial and community-acquired infections worldwide. Therefore, the detection of outbreaks of staphylococcal infections, as well as the identification of "successful" clones with enhanced virulence or increased ability to spread epidemically, is essential. The traditional methods of *S. aureus* typing have largely been replaced by molecular typing systems. These fulfill all of the criteria required for usefulness, including performance (typeability, reproducibility, stability, and discriminatory power) and convenience (rapidity, accessibility, ease of use, and ease of interpretation) (14, 18). Individually, however, they may suffer from various shortfalls.

Among the methods for molecular typing of *S. aureus* isolates, pulsed-field gel electrophoresis (PFGE) is considered to be the gold standard. Unfortunately, this method is technically demanding and time-consuming. Moreover, achieving full reproducibility between laboratories is challenging, thus making data comparison difficult (22). However, successful efforts at intercenter standardization of protocols were achieved, therefore allowing the development of multicenter methicillin-resistant *S. aureus* (MRSA) surveillance networks (2, 12, 13).

Traditional PCR-based techniques, in comparison to PFGE, are easier, faster, and increasingly less expensive to perform but also have several limitations. In the cases of a randomly amplified polymorphic DNA (RAPD) assay and a repetitive element sequence-based PCR (rep-PCR) analysis, the major drawback relates to insufficient standardization and low laboratory-to-laboratory reproducibility (3, 21). The PCR-based locus-specific restriction fragment length polymorphism technique suffers from limited discriminatory power as was shown by coagulase gene analysis (1). Methods which utilize DNA sequencing, including multilocus sequence typing (MLST) (5) and *spa* typing (8, 10), have a good discriminatory power and full portability of data, but their cost is a limiting factor for most laboratories.

During the past few years, many efforts have been made to develop new PCR-based typing methods for *S. aureus*. Among these methods is a triplex PCR aimed to amplify fragments of *spa*, *coa*, and the hypervariable region adjacent to the *mecA* gene (HVR) (17, 24), as well as restriction profile analysis of the repetitive element called STAR (STAR-RP) (15). Recently, a multiplex PCR method for multiple-locus variable-number tandem repeat (VNTR) analysis (16) has been developed. In our previous publications, we called this method multiple-locus VNTR analysis, but in fact, this is a "fingerprinting" approach, since it does not allow an analysis of the exact number of repeats in the obtained amplification products and determining which band corresponds to which PCR target is impossible. The resulting data cannot be compared between different laboratories. Therefore, we would like to rename this method multiple-locus VNTR fingerprinting (MLVF). The simultaneous analysis of the variation in number of repeat units in seven individual genes (*sspA*, *spa*, *sdrC*, *sdrD*, *sdrE*, *clfA*, and *clfB*) endows MLVF with a discriminatory power comparable to PFGE (11). On the basis of PFGE interpretation rules by Tenover et al. (20), criteria for clustering MLVF patterns have been suggested (11). Isolates classified into the same MLVF cluster differing by up to six DNA fragments, among a maximum of 14 bands, differed by no more than 6 bands in PFGE analysis. However, outbreak analysis and short-time interhospital spread of a highly clonal organism such as *S. aureus* demand more stringent criteria in MLVF interpretation. In more stringent conditions, the MLVF clusters grouped isolates that differed by up to four bands, which reflected up to three bands of difference between two PFGE patterns. Recently, Francois and colleagues (6) automatized and expanded MLVF with additional virulence genes containing repeat units (*fnbA*, *fnbB*, and *cna*).

Recently developed PCR-based typing methods (MLVF, expanded MLVF, triplex PCR [*coa*, *spa*, and HVR], and STAR-RP) have been poorly evaluated in comparison to the well-established assays (RAPD PCR and rep-PCR). The new methods require careful assessment of their potential for typing *S. aureus* strains, and there is a need to identify the most

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TABLE 1. Origin, methicillin phenotype, and typing results for 59 *S. aureus* isolates[f]

Isolate	Country/yr	Source (hospital designation)	PFGE type[a]	MLVF type[b]	Triplex PCR type	STAR-RP (AluI/Tru9I) type[c]	MLST type[a]	*spa* type[a]	Methicillin phenotype (*S. aureus* type)[d]
C115[e]	Poland/1994	A	A1	1	1	1 (1/1)	45	t015	HeMRSA
2688/98[e]	Poland/1998	B	A2	2	1	1 (1/1)	45	t015	HeMRSA
A005a[e]	Poland/1992	C	A4	3	2	1 (1/1)	45	t390	MSSA
MR89[e]	Poland/1992	D	B1	4	3	2 (2/2)	239	t037	HoMRSA
3121[e]	Poland/1996	E	B3	5	3	2 (2/2)	239	t388	HoMRSA
MR11	Poland/1992	D	B7	6	3	2 (2/2)	239	t037	HoMRSA
MR1064[e]	Poland/1992	F	B8	7	4	3 (3/3)	239	t037	HoMRSA
N98[e]	Poland/1995	C	B13	8	5	3 (3/3)	239	t037	HoMRSA
A005b[e]	Poland/1994	G	B28	9	3	3 (3/3)	239	t037	HoMRSA
H390[e]	Poland/1994	H	B45	10	3	2 (2/2)	239	t388	HoMRSA
1791/97[e]	Poland/1997	I	C1	11	6	3 (3/3)	157	t030	HoMRSA
1794/97	Poland/1997	I	C2	11	6	4 (4/4)	157	t030	HoMRSA
1807/97	Poland/1997	I	C4	11	6	4 (4/4)	157	t030	HoMRSA
2689[e]	Poland/1998	B	C7	12	7	2 (2/2)	239	t037	HoMRSA
MR1003[e]	Poland/1992	J	C8	11	6	3 (3/3)	239	t030	HoMRSA
N104a[e]	Poland/1995	G	C10	11	3	3 (3/3)	157	t030	HoMRSA
3028[e]	Poland/1996	K	D1	13	8	2 (2/2)	247	t052	HoMRSA
2233/97[e]	Poland/1997	L	D2	13	8	2 (2/2)	336	t052	HoMRSA
2234b/97	Poland/1998	L	D2	13	8	2 (2/2)	336	t052	HoMRSA
2255/98	Poland/1998	L	D2	13	8	2 (2/2)	336	t052	HoMRSA
2258/98	Poland/1998	L	D2	13	8	2 (2/2)	336	t052	HoMRSA
2577/98	Poland/1998	L	D2	13	8	2 (2/2)	336	t052	HoMRSA
303/00[e]	Poland/2000	M	D12	13	8	2 (2/2)	247	t052	HoMRSA
2260/98	Poland/1998	L	D13	13	8	2 (2/2)	247	t052	HoMRSA
BN4[e]	Poland/1996	K	E	14	9	5 (5/5)	1	t159	HeMRSA
MR80[e]	Poland/1992	D	F1	15	1	6 (3/6)	5	t053	HeMRSA
2700[e]	Poland/1998	B	F2	15	1	6 (3/6)	5	t053	HeMRSA
MR52	Poland/1992	D	F3	15	1	6 (3/6)	5	t053	HeMRSA
MR5	Poland/1992	D	F5	16	1	6 (3/6)	5	t053	HeMRSA
MR84	Poland/1992	D	F6	16	1	6 (3/6)	5	t053	HeMRSA
MR29	Poland/1992	D	F7	15	1	6 (3/6)	5	t053	HeMRSA
MR31	Poland/1992	D	F7	15	1	6 (3/6)	5	t053	HeMRSA
MR24	Poland/1992	D	F8	17	1	6 (3/6)	5	t053	HeMRSA
N39[e]	Poland/1995	K	F8	18	10	6 (3/6)	5	t389	HeMRSA
MR44	Poland/1992	D	F10	19	1	6 (3/6)	5	t053	HeMRSA
MR63	Poland/1992	D	F10	16	1	6 (3/6)	5	t053	HeMRSA
MR27	Poland/1992	D	F13	15	1	6 (3/6)	5	t053	HeMRSA
1899/96[e]	Poland/1996	E	G1	20	11	2 (2/2)	80	t044	HeMRSA
2838/98	Poland/1996	E	G2	21	11	2 (2/2)	80	t044	HeMRSA
MR1010a[e]	Poland/1992	D	G3	22	11	2 (2/2)	80	t044	HeMRSA
B098[e]	Poland/1994	N	J	23	12	3 (3/3)	239	t037	HoMRSA
771[e]	Poland/1997	O	K2	24	3	2 (2/2)	239	t037	HoMRSA
2690[e]	Poland/1998	B	K3	25	4	4 (4/4)	239	t387	HoMRSA
MR76[e]	Poland/1992	D	K5	26	13	3 (3/3)	239	t037	HoMRSA
J405[e]	Poland/1994	P	K14	24	3	3 (3/3)	239	t037	HoMRSA
MR47	Poland/1992	D	K16	27	14	2 (2/2)	239	t037	HoMRSA
2956[e]	Poland/2001	Q	N2	28	15	7 (6/7)	30	t391	HeMRSA
EMRSA-16[e]	England/1992	R	N3	29	16	8 (7/8)	36	t018	HeMRSA
2684[e]	Poland/1998	K	N5	30	17	8 (7/8)	30	t019	MSSA
3301[e]	Slovenia/1998	S	O1	31	18	2 (2/2)	501	t037	HoMRSA
3497[e]	Bulgaria/1998	T	O2	32	19	2 (2/2)	239	t037	HoMRSA
3254[e]	Turkey/1996	U	P	33	20	3 (3/3)	239	t030	HoMRSA
3498[e]	Russia/1998	V	Q	34	21	9 (8/9)	8	t008	HoMRSA
3502[e]	Bulgaria/1998	W	R	35	22	2 (2/2)	1	t386	HeMRSA
3248[e]	Czech Republic/1996	X	S	36	23	8 (7/8)	30	t012	HoMRSA
3521[e]	Lithuania/1998	Y	T	37	21	9 (8/9)	8	t008	HoMRSA
NCTC 8325[e]			U	38	24	3 (3/3)	8	t211	
794[e]	Poland/1997	O	W	39	25	2 (2/2)	15	t084	MSSA
3483[e]	Slovenia/1998	Z	Y	40	26	6 (3/6)	228	t001	HeMRSA

[a] Results previously obtained by Malachowa et al. (11).
[b] The same results were produced by both multilocus VNTR typing methods, MLVF (11) and expanded MLVF (this study).
[c] Restriction profiles obtained using restriction endonucleases AluI and Tru9I.
[d] Abbreviations for methicillin phenotypes: MSSA, methicillin-susceptible *S. aureus*; HeMRSA, *S. aureus* heterogeneously resistant to methicillin; HoMRSA, *S. aureus* homogeneously resistant to methicillin.
[e] Epidemiologically unrelated isolates used for calculation of the discriminatory power.
[f] Numbers of patterns obtained for all isolates (total; $n = 59$) and for epidemiologically unrelated isolates (total; $n = 40$) are as follows: for PFGE, 52 and 40, respectively; for MLVF, 40 and 34; for triplex PCR, 26 and 25; for STAR-RP, 9 (8/9) and 9 (8/9); for MLST, 14 and 14; and for *spa* typing, 20 and 20.

efficient PCR approach. In this study, we therefore evaluated six PCR-based typing methods and compared the results with those obtained by Malachowa et al. (11).

Fifty-nine nosocomial *S. aureus* strains, mostly MRSA isolates (Table 1), were obtained from the collection previously characterized by PFGE, MLVF, MLST, and *spa* typing (11). All isolates were subjected to six different PCR-based typing techniques that included (i) the multiple-locus variable-number tandem repeat-based method (expanded MLVF) with a primer mix to simultaneously amplify the parts of genes coding for serine protease V8 (*sspA*), protein A (*spa*), Ser-Asp-rich fibrinogen-binding proteins (*sdrCDE*), clumping factor A (*clfA*), clumping factor B (*clfB*), fibronectin-binding proteins (*fnbAB*), and collagen adhesin A (*cna*); (ii) triplex PCR (*coa*, *spa*, and HVR) with a primer mix to simultaneously amplify a part of the *coa* gene (coding for coagulase), a part of the *spa* gene (coding for protein A), and the hypervariable region adjacent to the *mecA* gene (17, 24); (iii) STAR-RP (15); and (iv) RAPD using the combination of primers EP007 and KAY1 (19); as well as two different rep-PCR methods, (v) inter-IS*256* (4) and (vi) rep-MP3 (23). The typing methods were performed with published protocols, with the exception of expanded MLVF, for which each PCR mixture was separated in 2% agarose Micropor GAMMA (Prona) gel by using a classical electrophoretic approach. Any two patterns produced by each of the six PCR genotyping procedures differing by one or more bands were considered distinct types. In order to test the reproducibilities of the PCR-based typing systems, we obtained the patterns from 20 isolates in three independent experiments with different DNA preparations. Reproducibility was defined as the ability to yield identical PCR patterns in testing the same isolate during different experiments. It was expressed as the percentage of isolates that gave the same profile in separate experiments. The discriminatory power of each PCR-based fingerprinting method was estimated by the number of identified patterns among 40 epidemiologically unrelated isolates (Table 1) and by Simpson's index of diversity (9). Ninety-five percent confidence intervals (CI_{95}) for discriminatory indices were calculated as previously described (7). Nonoverlapping confidence intervals were regarded as indicating statistically significant differences in discriminatory power (7). The calculation of intermethod concordance was conducted as described before (11).

Fifty-nine isolates of *S. aureus* were typeable by all the methods used in this study. Analysis of reproducibility was further performed three times on the reextracted total DNA of 20 isolates. An excellent reproducibility (100%) was observed for the expanded MLVF, triplex PCR, and STAR-RP methods, whereas reproducibilities of inter-IS*256* (85%), RAPD (80%), and rep-MP3 (75%) were lower. The intensity of the HVR amplicon in triplex PCR approach varied in different runs, but this, however, did not compromise the reproducibility of the method.

We further analyzed only those methods whose reproducibility was 100%. The expanded MLVF approach was the most discriminatory PCR-based method in this study, but in the case of triplex PCR, a difference was not statistically significant because of overlapping 95% confidence intervals (0.98 [CI_{95}, 0.98 to 1.00] and 0.96 [CI_{95}, 0.94 to 0.98]). The discriminatory power of STAR-RP was only 0.80 (CI_{95}, 0.74 to 0.86), and STAR-RP differed substantially from other PCR typing methods tested. Expanded MLVF distinguished 34 patterns that exactly matched the 34 MLVF profiles obtained previously (11). None of the PCR methods tested was able to distinguish all of the epidemiologically unrelated isolates (Table 1). However, only expanded MLVF could distinguish all isolates with distinct PFGE types (from types A to Y). Also, expanded MLVF was the only method which could differentiate between all *spa* types. In other words, different PFGE or *spa* types translated into different MLVF patterns.

TABLE 2. Correlation between typing methods for *S. aureus*

Method	Correlation between methods (%)					
	PFGE	MLVF[b]	Triplex PCR	STAR-RP	MLST	*spa* typing
PFGE	100					
MLVF[b]	72.9[a]	100				
Triplex PCR	52.5	71.2	100			
STAR-RP	23.7	39.0	50.8	100		
MLST	32.2[a]	44.1[a]	62.7	59.3	100	
spa typing	42.4[a]	62.7[a]	76.3	57.6	72.9[a]	100

[a] Results previously obtained by Malachowa et al. (11).
[b] The same results were produced by both multilocus VNTR typing methods, MLVF (11) and expanded MLVF (this study).

Concordance between the methods evaluated in this study ranged from 23.7 to 76.3% (Table 2), when all isolates from the collection were studied. The best concordances of results were found for *spa* typing with triplex PCR (76.3%) and MLST (72.9%) as well as expanded MLVF with PFGE (72.9%) and triplex PCR (71.2%). Overall, there was a high degree of concordance between the results obtained by the typing techniques with similar discriminatory abilities. In contrast, the least concordance values were observed between the typing methods which displayed different discriminative potentials, such as PFGE and STAR-RP (23.7%) or PFGE and MLST (32.2%).

Reproducibility is one of the most important parameters of any bacterial typing method. Among the PCR-based typing methods with high discriminatory power, expanded MLVF and triplex PCR had excellent reproducibilities (100%). Therefore, these approaches allow reliable comparisons across large numbers of assays and for comparing archived fingerprint patterns.

The triplex PCR and expanded MLVF approaches revealed very good concordances with *spa* typing, 76.3% and 62.7%, respectively. Both PCR methods utilize the VNTR region of the *spa* gene. Despite the fact that there was better concordance between triplex PCR and *spa* typing, only expanded MLVF could distinguish all *spa* types. Triplex PCR in some cases produced the same pattern for different *spa* types (triplex PCR types 1, 3, and 4). Moreover, better concordance was found between PFGE and expanded MLVF (72.9%) than between PFGE and triplex PCR (52.5%). Therefore, for large-scale surveillance, the MLVF methods are candidate screening tools for the identification of genetically identical isolates. From these, two or three representative isolates may be selected for further definitive characterization by *spa* typing in reference laboratories. The *spa* sequence data are unambiguous and electronically portable between laboratories. The searchable database on the Ridom SpaServer (www.ridom.de

/spaserver), along with StaphType software (8), can be used to increase our knowledge of genetic and epidemiological data from various geographic regions. Therefore, the combined use of MLVF or expanded MLVF and *spa* typing can benefit the monitoring of hospital-to-hospital strain transmission events and public health interventions on a huge scale.

We demonstrated that among the methods based on PCR, MLVF and expanded MLVF are most effective for typing large collections of MRSA strains. MLVF methods fulfill all the criteria for broadly used typing techniques, so they are rapid and inexpensive and not labor-intensive, with excellent discriminatory power and reproducibility. Adequate concordances with other highly discriminative typing methods, such as PFGE and especially *spa* typing, enable the MLVF approaches to be valuable techniques with an application to the investigation of outbreaks and for epidemiological tracing of *S. aureus*.

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