Evaluation of rep-PCR/DiversiLab versus PFGE and *spa* typing in genotyping methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Pulsed-field gel electrophoresis (PFGE) is the 'gold standard' for genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA); however, the DiversiLab (DL) system, based on rep-PCR, is faster, simpler and could be better adapted to daily routine hospital work. We genotyped 100 MRSA isolates using PFGE, DL, and *spa* typing, and evaluated the discriminatory power of each technique and the correlation between them by Simpson's index(SI) and adjusted Rand coefficient (ARI), respectively. The isolates were from clinical samples from eight hospitals in Extremadura (Spain) during 2010. DL separated the 100 MRSA into 18 patterns, with 69% of the isolates grouped into four predominant patterns. *spa* typing reported 17 *spa* types, classifying 69% of MRSA into two major types (t067 and t002). PFGE revealed the existence of 27 patterns, gathering 54% of MRSA into three pulse types (E8a, E7a and E7b). SI values were 0.819, 0.726, 0.887 and 0.460 for DL, *spa* typing, PFGE and CC-BURP, respectively. ARI values of DL over PFGE, *spa* typing and CC-BURP were 0.151, 0.321 and 0.071, respectively. DL has less discriminatory power than PFGE but more than *spa* typing. The concordance of DL with PFGE is low, primarily because DL does not discriminate between the three predominant MRSA pulse types in our environment.

KEY WORDS: Methicillin-resistant Staphylococcus aureus.
Molecular epidemiology.
Spain.

Introduction

Currently, the methods for genotyping and clonal analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) causing disease strains are considered valuable tools for tracking and limiting the spread of this organism.[1–3] It is estimated that each year in Europe MRSA causes 5400 deaths associated with health care, and the average increase in hospital stay length is between 10 and 26 days per patient.[4,5]

The ideal method for characterisation genotyping of MRSA should be easy and quick to perform, with sufficient discrimination power, high reproducibility, and all this, of course, at a low cost. It would also be useful if positive results obtained by this method in different reference laboratories or hospitals were comparable.[6]

The current reference method for genotyping MRSA, the pulsed-field gel electrophoresis (PFGE), does not meet all of these qualities because, although it has the highest discrimination power, it is slow, laborious and open to subjective interpretation, which requires the availability of experienced and trained staff to develop the technique.[7] However, the DiversiLab (DL) system, based on rep-PCR technology, is described in the literature as a fast and easy technique and is currently the only system genotyping microbial that is commercially available as a standardised kit.[8] It has demonstrated sufficient discrimination power and good reproducibility in surveillance studies of microbial species with nosocomial interest such as *Enterococcus faecium*,[9] *Pseudomonas aeruginosa*,[10] *Serratia marcescens*,[11] *Salmonella enteritidis*[12] and *Acinetobacter baumannii*.[13,14]

Our goal is to carry out a study of the clonality of MRSA infection produced in the region of Extremadura (Spain) using the methods DL, PFGE and *spa* typing. We will then calculate statistical indices to provide information about the power of agreement between the different methods and the discriminating power of each, paying particular attention to those reported by DL and PFGE. Based on this we will evaluate the benefits of having the DL technology for the detection of MRSA outbreaks in our hospitals.

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Materials and methods

Collection of bacterial isolates

Extremadura is a region located in the south-west of Spain, with a population of one million habitants divided into eight health areas, with each area having its own hospital. Between January and December 2010, all MRSA isolates from clinical specimens planted and processed in the routine work of each hospital were sent to the Microbiology Division of Merida Hospital. The isolates originated from general swabs, blood, urine, respiratory specimens, blood, catheter, and nasal swabs. The number of isolates collected at the end of 2010 is 309, of which we want to genotype a

representative sample of 100 isolates. We conducted stratified random sampling to select a sample of 100 isolates, which is proportionally reflected in each health area.

Genotyping by DiversiLab

MRSA isolates were typed using a DiversiLab kit (bioMérieux, Geneva, Switzerland) as recommended by the manufacturer. DNA was extracted from this sample using a method of column purification using the UltraClean microbiol DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA). The presence and concentration of DNA in the extraction product was estimated using the Nanodrop spectrophotometer ND-1000 (Witec, Lucerne, Switzerland). Amplification was performed using the DiversiLab *Staphylococcus* DNA fingerprinting kit (Spectral Genomics, Houston, TX, USA), which includes a rep-PCR MM1 buffer, primers specific for *S. aureus* (First Mix), and a set of positive and negative controls to ensure the correct development of the amplification reaction. The polymerase used was AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), supplied by the manufacturer together with the 10X PCR buffer. The reaction requires a substrate between 50–100 ng DNA. The thermocycler used was an Applied Biosystems 2720 thermal cycler with an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec, extension at 70°C for 90 sec, and a final extension at 70°C for 3 min. The analysis of the products of rep-PCR was performed using the B2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA), in which amplified fragments of varying sizes and quantities were separated and detected after capillary electrophoresis in Labchip. A chip can be loaded with 12 rep-PCR products of different samples or strains, reserving the last well for the molecular weight marker. The loaded volume of rep-PCR product was only 1 μL.

The comparison of results and percentages of similarity between isolates was calculated by the DiversiLab version 3.4 software, which determines the distance matrices according to the Pearson correlation coefficient and using unweighted arithmetic means for paired samples (UPGMA) to create the dendrograms. The reports were automatically generated and include dendrograms, electropherograms, virtual gel images and scatter plots. Isolates were classified according to their degree of relationship as 'indistinguishable', 'similar' or 'different', following the criteria recommended by the manufacturer. Those exhibiting similarity indices above 95% can be 'like' or 'indistinguishable'. Once this condition was met, we compared the profiles of the electropherograms, so that accurate profiles with no difference in peak, including no difference in intensity, were considered 'indistinguishable'. Indistinguishable isolates were classified within a clone or pattern (P). Those with a difference in a single peak were considered 'similar' and were classified within the same group (G). If ≥2 peaks were different, the isolate was classified as 'different' without any grouping option.

Genotyping by pulsed-field gel electrophoresis

The MRSA isolates were genotyped by PFGE after SmaI digestion of chromosomal DNA, prepared using the protocol described by Cuevas *et al*.[15] Analysis of the gels was performed according to the criteria of Tenover *et al*.[16] and a dendrogram was constructed with Molecular Analyst Software (Bio-Rad, Hercules, CA, USA) using the Dice correlation coefficient,[17] and the unweighted pair-group method with averages with a tolerance position of 0.8%. The PFGE type was assigned according to the criteria of Murchan *et al*.[18]

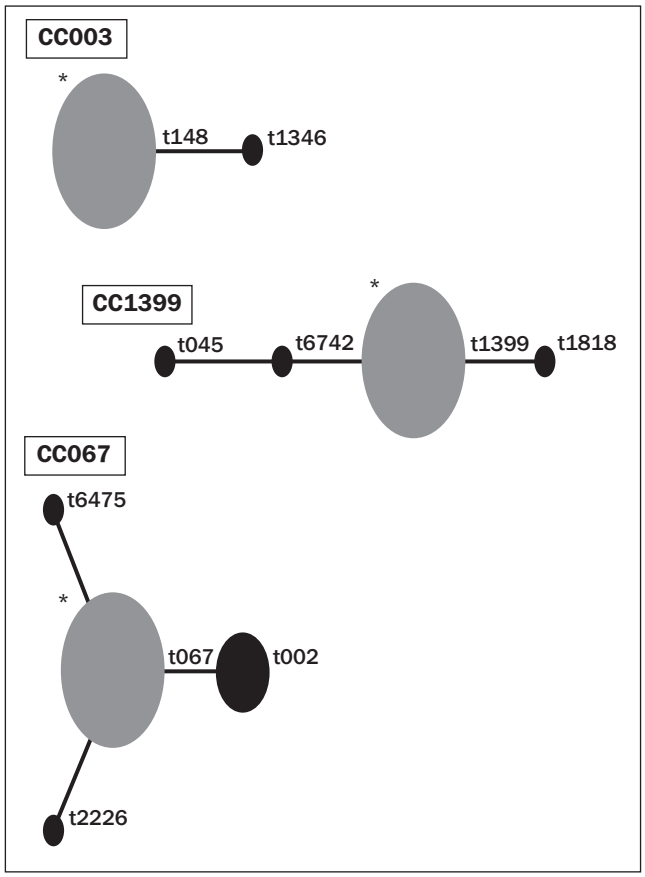

Fig. 1. Evolutionary relationship between the *spa* types that make up CC-BURP. *Genotype founder in grey.

Genotyping by **spa** *typing and* **spa** *clonal cluster analysis*

Both methods were carried out as described previously.[19] Nucleotide sequences were analysed using Ridom StaphType software and synchronised with SpaServer (www.spaserver.ridom.de). Clustering analysis into *spa* clonal complexes (*spa*-CC) was carried out using the BURP algorithm with default parameters.[20] It reported information about of the phylogenetic relation for each *spa*-type. The *spa* types with a length of less than five repeats were excluded from BURP analysis and were defined as 'excluded'. Those *spa* types that were not found in this study represented by a sufficient number of isolates were called 'no founder'. Those *spa* types that did not have sufficient similarity with any other *spa* type described to date were called 'singletons' Also, the StaphType software was used to infer the clustering analysis eBURST.

Calculation of discriminatory power and the concordance between molecular typing methods

The discrimination power of each genotyping technique is reported using Simpson's diversity index (SI), which reflects the probability that two unrelated isolates are assigned to different typing groups.[17] To compare two sets of results of different microbial typing methods, an objective measure of

Table 1. Association of genotypes obtained by DiversiLab and PFGE.

Group DiversiLab	Pattern Diversilab	Pulsotype (No. isolates)	Total isolates
1	1	E20 (2)	38
		E7a (10)	
		E7b (10)	
		E8a (5)	
		E8b (6)	
		sporadic (1)	
		sporadic (1)	
		sporadic (1)	
		sporadic (1)	
		sporadic (1)	
	2	E10 (3)	8
		E17 (1)	
		E8a (2)	
		E8b (1)	
		sporadic (1)	
	3	E10 (1)	2
		E16 (1)	
	4	E10 (1)	1
2	5	E20 (1)	8
		E7a (1)	
		E7b (5)	
		sporadic (1)	
	6	E7a (1)	5
		E8a (3)	
		sporadic (1)	
	7	E10	1
	8	E7b (2)	4
		E8a (2)	
3	9	sporadic (1)	4
		sporadic 1 (3)	
	10	sporadic (1)	1
	11	E13 (2)	2
	12	E13 (1)	1
4	13	A1 (1)	2
		E19 (1)	
	14	sporadic 3 (2)	2
	15	sporadic 2 (2)	3
		E8a (1)	
	16	E20 (1)	15
		E8a (11)	
		E8b (1)	
		sporadic (1)	
	17	**E8a (1)**	**2**
		sporadic (1)	
	18	sporadic (1)	1

agreement is needed. Several measures were developed for comparing two sets of partitions, taking different approaches to how partitions should be compared. For ease of interpretation, in this study we used the adjusted Rand coefficient and the Wallace coefficient. Rand and adjusted Rand are symmetric coefficients (i.e., they do not take into consideration which partition is considered the standard, while the Wallace coefficient does).

It is also important to note in this context that none of the partitions tested are considered the 'correct' partition in terms of microbial typing.[21] The adjusted Rand index (ARI) calculates the probability that two isolates are classified either in the same or in different genotypes genotype by both methods. The Wallace index set (WI) indicates the probability that two isolates classified in the same genotype for one method are also classified as the same by another method. A high WI value of method A with respect to method B means that the results obtained with method A can be predicted with a high probability to also be the results obtained with method B.[21,22]

To facilitate the calculation of these indices, we used web software (http://darwin.phyloviz.net/ComparingPartitions/index.php).[21–23] Confidence intervals of 95% of SI values were calculated as described by Grundmann *et al*.[24]

Results

DiversiLab

The 100 isolates were grouped into 18 different patterns or clones, with 38% of the isolates grouped in pattern P1. Patterns P2, P3 and P4, together with P1, form the group G1,

Table 2. Concordance of classification between *spa* typing, CC-BURP and eBURST.

spa type	No. isolates	CC-BURP (No. isolates)	MLST	eBURST (% isolates)
t067	47	CC067 (72)	ST125, ST5	CC5 (85)
t002	22			
t2226	2			
t6475	1			
t045	1	CC1399 (11)	ST146	
t1399	8			
t6472	1			
t1818	1			
t148	3	CC3 (4)	ST72	*mismatched
t1346	1			
t7284	1	Excluded (2)		*mismatched
t535	1			
t109	2	Singleton (11)		CC5 (85)
t032	3			CC22 (3)
t127	1			*mismatched
t008	4			CC8 (4)
t012	1			CC30 (1)

*mismatched: *spa* types that did not map with any clonal group eBURST.

which includes 49% of the isolates. Patterns P5 and P6 form G2, with 13% of the isolates. There were two other groups, G3 and G4, with 5% and 4% of the isolates, respectively. The rest of the patterns are not grouped with any other pattern, and are a minority (1–4% of isolates) except P16 with 15% of the isolates (Table 1).

spa *typing,* spa-*CC analysis and assigning eBURST*

spa typing differentiated the collection into 17 different *spa* types, among which *spa* types t067 and t002 were predominant, grouping 47% and 22% of MRSA *spa* types, respectively. The remaining 15 *spa* types represented very low frequencies ranging from 1% to 8%. The BURP tool, part of the Ridom StaphType software, revealed three clonal groups: CC067 (72%) consisting of t067, t002, t2226 and t6475; CC1399 (11%) consisting of t045, t1399, t6472 and t818; and CC3 (4%) consisting of t148 and t1346. Two *spa* types were excluded from BURP analysis and classified as 'excluded'. Another 11 isolates, spread over five *spa* types that did not have sufficient similarity to any other *spa* type to form a clonal group, were classified as 'singleton' (Table 2). The Ridom StaphType software has a tool that easily represents the evolutionary relationship between the *spa* types of the same clonal group CCBURP. Applied to our three different clusters, we obtained the representation shown in Figure 1. As shown, the *spa* types t148, t1399, t067 were identified as the founders and ancestral genotypes of clusters CC3, CC1399 and CC067, respectively. The eBURST algorithm reflects a close phylogenetic relationship among the vast majority of isolates genotyped, because the *spa* type clusters of CC1399 and CC067, besides the two isolated t109, were classified in the group of eBURST clonal CC5, which represented 85% of the total. The remainder are associated with minor clonal groups, namely the CC22 (*spa* type t032, 3%), the CC8 (constituted by t008, 3%) and CC30 (isolated t012; 1%) (Table 2). Tables 3 and 4 display the association of

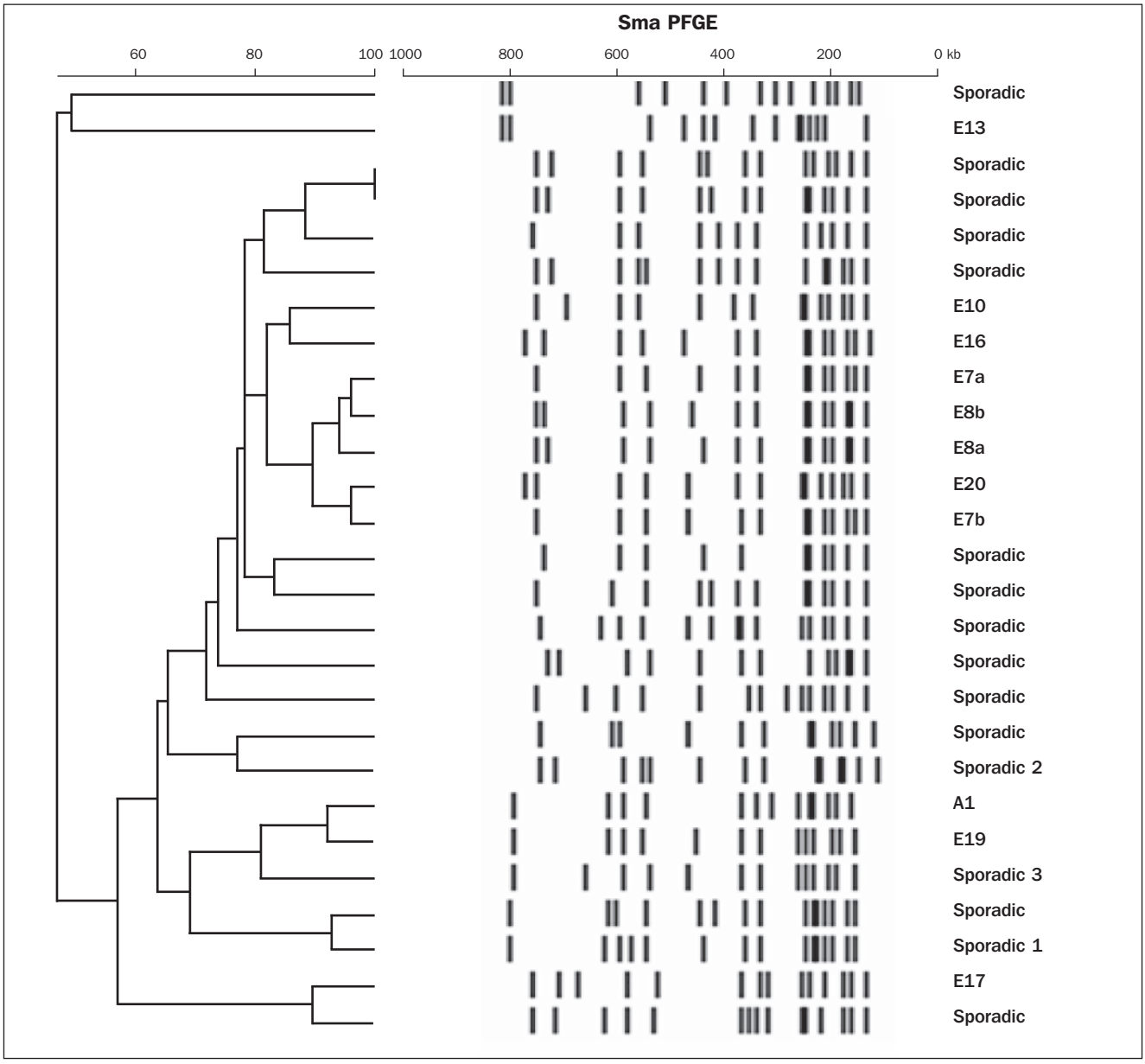

Fig. 2. Dendrogram showing the group relationship between different pulse types described and a representation of the pattern of bands corresponding to each pulse type

genotypes obtained by DL and PFGE regarding genotypes obtained by *spa* typing, respectively.

Pulsed-field gel electrophoresis

Using PFGE resulted in 27 pulse types. Of these, 54% were grouped into only three genotypes: E8a (25%), E7b (17%) and E7a (12%). The remaining 46% are distributed across 24 distinct genotypes. Of these, eight genotypes corresponded to a pattern of bands assigned a name and described in previous studies: E8b (8%), E10 (6%), E20 (4%), E13 (3%), A1 (1%), E16 (1%), E19 (1%), E17 (1%). Up to 16 patterns with sporadic profiles were represented by only one isolate, with the exception of three patterns that repeated in more than one isolate. These pulse types were called 'sporadic', followed by a number in the order of appearance. We observed Sporadic 1 (3% of isolates), Sporadic 2 (3% of isolates), and Sporadic 3 (2% of the isolates). Other sporadic patterns obtained in each single isolate were not assigned a suffix-specific identification (Table 1, Fig. 2).

Discrimination and concordance powers of the genotyping methods

We observed that PFGE presented the greatest power of discrimination, followed by DL and then *spa* typing. The CC-BURP presented a very low value SI relative to the other three methods (Table 5). In the quantitative analysis of agreement, reflected through the ARI, we saw that the best value was obtained for *spa* typing and CC-BURP, followed by the value obtained between *spa* typing and DL (Table 6). The coefficients obtained for the WI were all quite low, with the exception of *spa* typing using CCBURP. Thus, the values shown in Table 7 indicate that the ability to predict the results of the PFGE with either *spa* typing or DL is almost null.

Discussion

Our group confirms that genotyping with DL is fast and relatively easy to do from a technical standpoint, as previous studies concluded.[6,7,25,26] While PFGE required 3–5 days to obtain results, DL results can be achieved in a single day. Thanks to its commercial kit format and supporting computer software (included in a web page), DL provides results that are standardisable between laboratories and generate useful reports for clear understanding. However, we believe that the first step of the technique, the DNA extraction, is rather long and laborious and could be replaced by an automated technique. We also understand that the DL system currently lacks a universal nomenclature regarding MRSA clones, defined (as with PFGE and *spa* typing) to facilitate the understanding of inter-laboratory results; this is a major drawback of the DL system. Thus, we believe that, in the future, laboratories that choose this method to genotype MRSA should agree on a standard nomenclature for major clones.

The agreement between the different methods tested, evaluated quantitatively by calculating the ARI, shows a slight correlation between the DL and CC-BURP grouping. For DL, only 51% of MRSA isolates belonging to the major clonal group, CC067, are ranked in Group 1, 18% in Group 2, and the rest are represented in up to five different DL patterns. In contrast, there is a perfect agreement between the CC3 of *spa* typing and Group 3 of DL, and we found that all t008 isolates are classified as Group 4 DL. These two concordance cases represent a small percentage of the isolates genotyped, hence the Rand index value between the *spa* typing and DL methods is only 0.321, although this value is the highest of those obtained in the study of pairs of concordance between PFGE, *spa* typing and DL. The discrete ARI value between DL and *spa* typing is supported by a WI value of 0.435, denoting that the ability to predict the results of *spa* typing using those obtained by the DL technology is quite limited.

Table 3. Association of genotypes obtained by DiversiLab and *spa* typing.

Group DiversiLab	Pattern DiversiLab	*spa* type (No. isolates)	CC-BURP	Total isolates
1	1	t067 (29)	CC067	38
		t1399 (7)	CC1399	
		t7284 (1)	Excluded	
		t1818 (1)	CC1399	
	2	t535 (1)	Excluded	8
		t067 (3)	CC067	
		t109 (2)	Singleton	
		t002 (2)	CC067	
	3	t067 (1)	CC067	2
		t002 (1)		
	4	t002 (1)	CC067	1
2	5	t067 (6)	CC067	8
		t2226 (1)		
		t6475(1)		
	6	t1399 (1)	CC1399	5
		t002 (1)	CC067	
		t067 (3)		
	7	t067 (1)	CC067	1
	8	t067 (3)	CC067	4
		t2226 (1)		
3	9	t1346 (1)	CC3	4
		t127 (1)	Singleton	
		t148 (2)	CC3	
	10	t148 (1)	CC3	1
	11	t032 (2)	Singleton	2
	12	t032 (1)	Singleton	1
4	13	t008 (2)	Singleton	2
	14	t008 (2)	Singleton	2
	15	t002 (3)	CC067	3
	16	t002 (12)	CC067	15
		t067 (1)		
		t045 (1)	CC1399	
		t6472 (1)		
	17	t002 (2)	CC067	2
	18	t012 (1)	Singleton	1

When evaluating the correlation between DL and the gold standard for MRSA typing, PFGE, we find that it is even lower than that observed between DL and *spa* typing. None of the three predominant pulse types, E8a, E7b and E7a, are associated with a DL pattern, even at 60% of the isolates. The DL pattern most associated with pulse type E8a is 16, but it only contains 11 of the 25 isolates described in E8a. The rest are rated up to seven different patterns (P1, P2, P6, P8, P15, P16 and P17). Most of the E7b isolates are classified in P1 by DL (10 of 17 isolates); however, a group of five isolates are genotyped as P5 (which does not belong to Group 1) and two are classified as P8.

The clearest evidence of this lack of agreement is that, within the dominant pattern DL, the P1 (38 isolates), there are isolates belonging to 10 different pulse types. If we evaluate the pulse types represented by more than two isolates (nine pulse types), we see that there is a single DL pattern containing all isolates (all three isolates typed as Sporadic 1 are classified as P9 by DL). The rest of these pulse types have spread their isolates across various DL patterns. This clear lack of agreement is ratified with an ARI between DL and PFGE of only 0.151, coinciding with the findings of Babouee *et al*.[27] (ARI = 0.083) and Witt *et al*.,[26] who described (in the latter case only qualitatively) a discrepancy between the results obtained by both methods.

Recent studies[25,28] characterise DL as a good initial screening method in an outbreak of MRSA or even for small epidemiological studies. However, as the same DL pattern of isolates belong to the predominant pulse types E8a, E7b and E7a (e.g., P1 has in numerous isolates of each of these three major pulse types, or P8 is composed of two isolated E7b and two of E8a), our group advises against using the DL system in our environment for molecular typing of MRSA, even for the initial screening of a hospital outbreak.

Despite the clear discrepancy between the results obtained by PFGE and DL, both show a similar discrimination power. Looking at the SI values of the methods tested, PFGE produces the highest (0.887) and remains the most discriminatory technique. However, DL, with an SI of 0.819, close to that of PFGE and higher than *spa* typing (0.726), presents a remarkable discriminatory ability.

Table 4. Association between the results obtained by PFGE, *spa* typing, BURP CC-clonal grouping and assignment eBURST.

Pulsotype	*spa* type (No. isolates)	CC-BURP	eBURST
E8a	t002 (13)	CC067	CC5
	t067 (10)		
	t045 (1)	CC1399	
	t1399 (1)		
E7b	t067 (9)	CC067	
	t2226 (2)		
	t6475 (1)		
	t1399 (5)	CC1399	
E7a	t067 (11)	CC067	
	t1399 (1)	CC1399	
E8b	t067 (7)	CC067	
	t6472 (1)	CC1399	
E10	t002 (4)	CC067	CC5
	t067 (1)		
	t535 (1)	Excluded	*mismatched
E20	t067 (4)	CC067	CC5
E16	t067 (1)		
sporadic	t067 (1)		
sporadic	t067 (1)		
sporadic	t067 (1)		
sporadic	t067 (1)		
sporadic	t067 (1)		
sporadic	t002 (1)		
sporadic	t067 (1)		
sporadic 2	t002 (3)		
sporadic	t1818 (1)	CC1399	
sporadic	t109 (1)	Singleton	
E17	t109 (1)		
sporadic 1	t148 (3)	CC3	*mismatched
sporadic	t148 (1)		
sporadic	t7284 (1)	Excluded	*mismatched
sporadic 3	t008 (2)	Singleton	CC8
A1	t008 (1)		
E19	t008 (1)		
E13	t032 (3)		CC22
sporadic	t012 (1)		CC30
sporadic	t127 (1)		*mismatched

*mismatched: *spa* types that did not map with any clonal group eBURST

Table 5. Discrimination power of each genotyping method.

Genotyping method	No. islolates tested	No. of types	No. isolates classified in the majority type	SI (CI 95%)
DL	100	18	38	0.819 (0.757–0.881)
spa typing	100	17	47	0.726 (0.650–0.803)
PFGE	100	27	25	0.887 (0.850–0.923)
CC-BURP	100	5	72	0.460 (0.347–0.573)

IS: Simpson's Diversity Index. CI: confidence interval.

Table 6. Concordance between typing methods using the Rand coefficient.

Method	Rand coefficient			
	PFGE	*spa* typing	CC-BURP	DL
PFGE				
spa typing	0.136			
CC-BURP	0.094	0.486		
DL	0.151	0.321	0.071	

Table 7. Concordance between typing methods using the Wallace coefficient.

Method	Wallace Index (CI 95%)			
	PFGE	*spa* typing	CC-BURP	DL
PFGE		0.269 (0.156–0.381)	0.477 (0.234–0.720)	0.206 (0.087–0.324)
spa typing	0.091 (0.033–0.149)		1.000 (1.000–1.000)	0.255 (0.098–0.411)
CC-BURP	0.052 (0.000–0.111)	0.321 (0.201–0.441)		0.042 (0.000–0.130)
DL	0.119 (0.047–0.192)	0.435 (0.241–0.629)	0.224 (0.000–0.509)	

These results are similar to those provided by Witt *et al*.[26] (SI=0.905 for PFGE and SI=0.860 for DL). In fact, in situations where PFGE is unavailable, based on the results of the present study, we would recommend using DL over *spa* typing for genotyping of MRSA, not just for greater discriminative power, but also because, judging by the values of ARI and WI obtained, DL has greater concordance and greater predictive power of the types with PFGE than *spa* typing. This recommendation disagrees with the two previously mentioned studies, which reported a nearly identical discrimination power for *spa* typing and PFGE,[27,29] and conclude that MRSA isolates that share the same DL pattern and PFGE could be distinguished by *spa* typing.[28]

A clear example that PFGE is the most discriminatory method is the case of t008, where four isolates were classified by DL in the same group; however, PFGE differentiated three different pulse types: Sporadic 3 (two isolates), A1 (one isolate) and E19 (one isolate).

In conclusion, with respect to the DL system for molecular typing of MRSA, which we found technically quick and easy to perform, our study results disagree with those of PFGE in relation to major MRSA clones in Extremadura and Spain, classifying in the same DL pattern those isolates belonging to different pulse types prevalent in our MRSA population, despite showing a respectable discrimination power even greater than *spa* typing. □

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