Original

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An analysis of the association between genotype and antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* clinical isolates

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ABSTRACT

Genotyping methods are useful resources for the surveillance, detection, prevention and control of multidrug-resistant nosocomial agents, such as methicillin-resistant *Staphylococcus aureus* (MRSA). An understanding of the association between genotype and antibiotic susceptibility in MRSA clones may be useful in the surveillance of MRSA and to avoid inappropriate treatment future resistance. We genotyped MRSA clinical isolates from the Extremadura region of Spain using pulsed field electrophoresis (PFGE) and analyzed the spectrum of antibiotic susceptibility for each isolate to determine whether resistance is associated with specific genotypes. PFGE revealed six major genotypes: E8a (25%), E7b (17%), E7a (12%), E8B (8%), E10 (6%), and E20 (4%). Isolates with the genotypes E8a and E10 exhibit higher resistance ratios for levofloxacin than isolates with the other major pulsotypes. Similar results were obtained for isolates with the E20 pulsotype with respect to mupirocin. Although we identified no vancomycin-, tigecycline-, linezolid- or daptomycin-resistant strains, we observed significant differences in the mean MIC values obtained for some of these drugs among the major genotypes. Specifically, isolates with the E7b, E8b, and E20 genotypes have significantly higher MICs of tigecycline, vancomycin and linezolid, respectively, than the most sensitive pulsotypes. Isolates with the E8b profile also exhibit a significantly higher rate of reduced vancomycin susceptibility (RVS) (i.e., MIC between 1 and 2 mg/L) than clones with the E10 and E8a profiles. In conclusion, we report associations between genotype and antibiotic sensitivity that should be considered in programs for monitoring and controlling MRSA in health care settings.

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Análisis de la asociación entre genotipos y resistencia a múltiples antimicrobianos en aislados de *Staphylococcus aureus* resistente a meticilina

RESUMEN

Los métodos de genotipaje son un recurso útil para la vigilancia, detención, prevención y control de agentes nosocomiales con multirresistencia antibiótica, como es *Staphylococcus aureus* resistente a meticilina (SARM). Nuestro grupo lleva a cabo el genotipaje mediante Electroforesis en Campo Pulsado (PFGE) de cepas SARM productoras de infección en la región de Extremadura (España), y realiza un estudio de asociación de los clones obtenidos, con respecto a la sensibilidad antibiótica mostrada por las cepas genotipadas, con el objetivo de conocer si existen resistencias que puedan asociarse específicamente a genotipos concretos. PFGE revela la existencia de 6 genotipos mayoritarios: E8a (25%), E7b (17%), E7a (12%), E8b (8%), E10 (6%), E20 (4%). A través del test Exacto de Fisher determinamos que los genotipos E8a y E10 se inclinan hacia ratios de resistencia mayores para levofloxacino en comparación a los mostrados por otros pulsotipos mayoritarios. De manera análoga ocurre para el pulsotipo E20 con mupirocina. Aunque no se encuentran cepas resistentes para vancomicina, tigeciclina, linezolid y daptomicina, encontramos en los tres primeros, diferencias significativas en el valor medio de CMI obtenido en los diferentes genotipos mayoritarios. Concretamente E7b, E8b y E20 presentan CMI significativamente más altas con respecto a tigeciclina, vancomicina y linezolid, respectivamente, en relación a los pulsotipos más sensibles. Además el perfil E8b muestra un mayor número de cepas con sensibilidad disminuida a vancomicina (SDV) (CMI entre 1 y 2 mg/L) que los clones E10 y E8a, de manera significativa. Creemos que esta información puede resultar útil en la vigilancia de la sensibilidad antibiótica de SARM en nuestro medio, para evitar tratamientos inadecuados y/o futuras resistencias.

INTRODUCTION

Increasing resistance of microorganisms to antimicrobial drugs is a growing global public health problem, particularly among microorganisms that cause nosocomial infections. Antimicrobial resistance leads to increased morbidity, mortality, and hospital costs[1-4]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the primary causes of nosocomial infections. Because MRSA infection typically occurs in patients with serious health conditions, it is considered a health care-associated infection[5,6]. The progressive increase of glycopeptide-resistant MRSA isolates[7,8] and isolates resistant to MRSA-specific antibiotics, such as daptomycin and linezolid[9-11], highlights the importance of infection control measures in healthcare settings. Thus, genotyping methods have become essential elements in epidemiological analyses, as powerful tools that complement the strategies used to fight the spread of infectious diseases and facilitate the implementation of new programs for the detection, control and prevention of outbreaks[12,13].

The aim of our study is to assess the antimicrobial sensitivity of MRSA strains causing infection in the Extremadura region of Spain and to study the clonality of these strains by genotyping them using pulsed field electrophoresis (PFGE), the gold standard method for molecular typing of MRSA strains. We also asked whether antibiotic resistance is associated with a particular genotype, a question that has not been addressed by previous studies. The phenomenon of resistance to vancomycin is particularly important. Although in our setting, MRSA strains exhibiting vancomycin resistance (VRSA) or intermediate resistance (VISA) have not been reported[14,15], strains with a MIC for vancomycin between 1 and 2 mg/L are frequently isolated. Treatment of these strains with vancomycin is frequently associated with an unsatisfactory clinical outcome[10,16,17].

MATERIAL AND METHODS

Collection of bacterial isolates. Extremadura is located in the southwest region of Spain, with a population of 1 million habitants. Extremadura is divided into eight Health Areas, and each Health Area has a hospital. Between January and December 2010, all MRSA isolates from clinical specimens processed during the routine work of each hospital were sent to the Division Microbiology at Merida Hospital. The strains originated from general swabs, blood, urine, respiratory specimens, catheter, and nasal swabs. A total of 309 isolates were collected, and we conducted stratified random sampling to select a sample of 100 strains, which proportionally reflected the eight Health Areas, for genotyping.

Antibiotic susceptibility study. Each isolate was subjected to a susceptibility analysis using the following antibiotics: gentamicin, tobramycin, levofloxacin, erythromycin, clindamycin, quinupristin/dalfopristin (QD), teicoplanin, fosfomycin, nitrofurantoin, fusidic acid, mupirocin, rifampin, and trimethoprim/sulfamethoxazole (SXT). Susceptibility was tested by microdilution in broth using the AST-588 card in the automated Vitek 2® system (Biomerieux, Geneva, Switzerland). For each isolate, E-test strips were used to determine the MICs for tigecycline, vancomycin, daptomycin and linezolid, which are specifically indicated for MRSA infections. For each strain, we prepared a microbial suspension in the range of 0.5-0.6 McFarland standard. The suspensions were plated on 15 cm plates containing MH medium and the cross-shaped E-test strips for the four antibiotics. The plates were incubated for 24h at 37°C, and the MIC was determined for each antibiotic.

Genotyping using PFGE. The MRSA isolates were genotyped using PFGE following SmaI digestion of chromosomal DNA, which was prepared using the protocol described by Cuevas et al.[18]. Analysis of the gels was performed according to the criteria described by Tenover et al.[19], and a dendrogram was constructed with Molecular Analyst Software® (Bio-Rad, Hercules, California, USA) using the Dice correlation coefficient20 and the unweighted pair-group method with averages with a tolerance position of 0.8%. A PFGE type was assigned to each isolate according to the criteria described by Vindel et al.[21].

Statistical analysis. Statistical tests, including the Chi-square test, Fisher's exact test, ANOVA, and Tukey's test, were performed using the SPSS 15.0. Software® (IBM, Armonk, New York, USA). The false discovery rate (FDR) test was performed using Statistical Software and Programming Language R 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

The molecular analysis of the 100 MRSA strains revealed 27 pulsotypes. The following three genotypes accounted for 54% of the isolates: E8a (25%), E7b (17%) and E7a (12%). The remaining 46% of the isolates were distributed among 24 distinct genotypes. Of these genotypes, 8 genotypes corresponded to patterns of bands that were previously described and assigned names: E8b (8%), E10 (6%), E20 (4%), E13 (3%), A1 (1%), E16 (1%), E19 (1%), and E17 (1%). We obtained up to 16 patterns with sporadic profiles that were represented by only a single isolate. Three sporadic patterns were observed in more than one isolate. These patterns were named "sporadic," followed by a number designating their order of appearance. Thus, we observed Sporadic 1 in 3% of isolates, Sporadic 2 in 3% of isolates, and sporadic 3 in 2% of isolates. The 16 sporadic patterns that were represented by only a single isolate were not assigned a specific identification suffix.

Tables 1 and 2 show the susceptibility ratios for the antibiotics tested using the Vitek® system for each of the described pulsotypes. The Chi-square test revealed significant differences in susceptibility and resistance ratios between different genotypes for gentamicin ($p = 0.001$), levofloxacin ($p = 0.001$) and mupirocin ($p = 0.001$). However, when a post-hoc pairwise comparison of genotypes was performed using the FDR method, no significant differences were observed between pulsotypes for these three antibiotics. The combination of this pairwise comparison with Fisher's exact statistic revealed the following important trends: a) Isolates with the most frequently observed genetic profile (i.e., E8a) and with profiles ESP1 and E10 have a significantly higher rate of levofloxacin

Table 1 **Susceptibility and resistance ratios obtained for each pulsotype (with more than one isolate) in antibiotics tested by microdilution in broth.**

Pulsotype	Total isolates	GNT		TBM			LVF			ERT		CLN		QN	FOS		NTR	FUS		RIF		SXT	MUP	
		S	R	S	R	I	S	R	I	S	R	S	R	S	S	R	S	S	R	S	R	S	S	R
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
E8a	25	100	0	16	84	0	0	60	40	44	56	76	24	100	92	8	100	100	0	100	0	100	100	0
E7b	17	100	0	12	88	0	0	94	6	12	88	88	12	100	88	12	100	100	0	100	0	100	94	6
E7a	12	100	0	8	92	0	0	100	0	33	67	58	42	100	75	25	100	100	0	100	0	100	100	0
E8b	8	88	12	25	63	12	0	100	0	25	75	50	50	100	88	12	100	88	12	88	12	100	100	0
E10	6	100	0	17	83	0	0	50	50	33	67	83	17	100	100	0	100	100	0	100	0	100	83	17
E20	4	75	25	25	75	0	0	75	25	0	100	75	25	100	50	50	100	100	0	100	0	100	50	50
ESP 1	3	100	0	0	100	0	0	33	67	67	33	100	0	100	67	33	100	100	0	100	0	100	100	0
ESP 2	3	100	0	33	67	0	0	67	33	33	67	33	67	100	100	0	100	100	0	100	0	100	100	0
E13	3	100	0	100	0	0	0	100	0	100	0	100	0	100	100	0	100	100	0	100	0	100	100	0
ESP 3	2	100	0	0	100	0	0	100	0	0	100	0	100	100	100	0	100	100	0	100	0	100	100	0

S: Susceptible, R: Resistant. GNT: Gentamicin, TBM: Tobramycin, LVF: Levofloxacin, ERT: Erythromycin, CLN: Clindamycin, QN: Quinupristin-Dalfopristin, FOS: Fosfomycin, NTR: Nitrofurantoin, FUS: Fusidic Acid, RIF: Rifampicin, SXT: Trimethoprim-sulfamethoxazole, MUP : Mupirocin. In bold, the resistance and susceptibility significant ratios by pairwise comparison with E.Fisher test.

Table 2 **Patterns of susceptibility antibiotic obtained in pulsotype represented by a single isolate, for antibiotics tested by microdilution in broth.**

Pulsotype	Total isolates	GNT	TBM	LVF	ERT	CLN	QN	FOS	NTR	FUS	RIF	SXT	MUP
A1	1	S	S	S	S	S	S	S	S	S	S	S	R
ESP	1	S	R	I	S	S	S	S	S	S	S	S	S
ESP	1	S	R	I	S	S	S	S	S	S	S	S	S
ESP	1	S	R	R	S	S	S	S	S	S	S	S	S
E19	1	S	R	R	S	S	S	S	S	S	S	S	S
ESP	1	S	S	R	R	S	S	S	S	S	S	S	S
ESP	1	S	S	R	R	S	S	S	S	S	S	S	S
ESP	1	S	S	S	R	R	S	S	S	S	S	S	S
ESP	1	R	R	R	S	S	S	S	S	S	S	S	S
ESP	1	S	R	R	R	S	S	S	S	S	S	S	S
ESP	1	S	R	R	R	S	S	S	S	S	S	S	S
E16	1	S	R	R	R	S	S	S	S	S	S	S	S
ESP	1	S	R	I	R	S	S	S	S	S	S	S	S
ESP	1	R	R	I	R	S	S	S	S	S	S	S	R
ESP	1	S	R	R	R	R	S	R	S	S	S	S	S
ESP	1	R	R	R	R	R	S	R	S	S	S	S	R
E17	1	R	R	R	R	R	S	R	S	S	S	S	R

S: Susceptible, R: Resistant. GNT: Gentamicin, TBM: Tobramycin, LVF: Levofloxacin, ERT: Erythromycin, CLN: Clindamycin, QN: Quinupristin-Dalfopristin, FOS: Fosfomycin, NTR: Nitrofurantoin, FUS: Fusidic Acid, RIF: Rifampicin, SXT: Trimethoprim-sulfamethoxazole, MUP : Mupirocin.

Table 3 Statistical parameter values for each genotype represented by more than one isolated, relative to the MIC (mg/L) of particularly suitable antibiotics for MRSA obtained by E-test.

		Tigecycline	Daptomycin	Vancomycin	Linezolid
	N	2	2	2	2
ESP 3	Maximum	0.125	0.094	1	0.5
	Mean	0.125	0.079	1	0.44
	N	6	6	6	6
E10	Maximum	0.19	0.19	1.5	2
	Mean	**0.142**	0.136	**1.2**	1.042
	N	3	3	3	3
ESP 2	Maximum	0.25	0.19	1.5	1.5
	Mean	0.167	0.147	1.2	1.083
	N	3	3	3	3
E13	Maximum	0.19	0.094	1	1
	Mean	0.19	0.094	0.9	0.917
	N	4	4	4	4
E20	Maximum	0.19	0.25	2	0.75
	Mean	0.19	0.15	1.6	**0.625**
	N	12	12	12	12
E7a	Maximum	0.25	0.38	2	2
	Mean	0.196	0.157	1.4	1.386
	N	17	17	17	17
E7b	Maximum	0.38	0.25	2	2
	Mean	**0.263**	0.153	1.4	**1.426**
	N	25	25	25	25
E8a	Maximum	0.38	0.19	1.5	2
	Mean	0.205	0.123	**1.3**	**1.12**
	N	8	8	8	8
E8b	Maximum	0.38	0.25	2	2
	Mean	0.236	0.177	**1.6**	**1.469**
	N	3	3	3	3
ESP 1	Maximum	0.25	0.19	1.5	2
	Mean	0.23	0.147	1.5	1.5

In bold, mean MIC significant by pairwise comparison with test Fisher's exact.

resistance (40, 67, and 50% of isolates with intermediate sensitivity, respectively) than isolates with the genetic profiles E7b and E7a (E8a-E7b, p = 0.016; E8a-E7a, p = 0.015; ESP1-E7b, p = 0.029; ESP1-E7a, p = 0.029; E10-E7b, p = 0.040; E10-E7a, p = 0.025). b) A comparison of isolates with the E20 genotype to isolates with different genotypes revealed that isolates with the E20 genotype have a significantly higher rate of mupirocin resistance than isolates with the genotypes E8a (p = 0.015) and E7a (p = 0.050).

Table 4 MIC values for particularly suitable antibiotics for MRSA infection, obtained for the pulsotypes represented by a single isolate.

	MIC (mg/L)			
	Tigecycline	Daptomycin	Vancomycin	Linezolid
A1	0.125	0.125	1	0.75
E16	0.25	0.19	1.5	2
E17	0.25	0.19	1.5	2
ESP	0.25	0.25	1.5	1.5
ESP	0.25	0.094	1	0.75
ESP	0.25	0.125	1.5	1.5
ESP	0.25	0.25	1.5	2
ESP	0.19	0.125	1.5	1
ESP	0.19	0.094	1	0.75
ESP	0.19	0.125	1.5	0.75
ESP	0.125	0.125	1	0.75
ESP	0.19	0.125	1	0.5
ESP	0.125	0.125	0.38	0.5
E19	0.125	0.125	1	0.38
ESP	0.25	0.125	1	2
ESP	0.25	0.094	1	0.75
ESP	0.19	0.125	1	0.5

Differences in susceptibility and resistance ratios between pulsotypes were not statistically significant for the remaining antibiotics tested using the Vitek 2® system: tobramycin (p = 0.622), erythromycin (p = 0.150), clindamycin (p = 0.208), fosfomycin (p = 0.257), fusidic acid (p = 0.993), and rifampicin (p = 0.993). It is worth noting that no resistant strains were observed for nitrofurantoin, QD, and SXT.

We observed highly variable sensitivity profiles for the set of pulsotypes represented by a single isolate (table 2). These profiles ranged from genotypes that were sensitive to most of the tested antibiotics, such as A1 (i.e., only mupirocin-resistant) and E19 (i.e., only tobramycin- and levofloxacin-resistant), to genotypes that were resistant to most of the test antibiotics, such as E17 (i.e., resistant to gentamicin, tobramycin, levofloxacin, erythromycin, clindamycin, mupirocin, and fosfomycin).

The E-test method for the determination of MICs for antibiotics that are particularly suitable for MRSA infection in each of the genotyped MRSA isolates failed to identify strains resistant to linezolid, tigecycline, daptomycin or vancomycin (tables 3 and 4). However, 61% of the isolates were characterized as RVS strains (table 5).

To evaluate differences in the MICs for vancomycin and tigecycline between different genotypic profiles, ANOVA was used. The results of this test confirm that these differences are

Table 5 **Proportions of RVS isolates on each majority genotype.**

		Vancomycin MIC (mg/L)	
		<1	>1
E10	nº (%)	4 (66.7)	**2 (33.3)**
	% of total	4	2
E7a	nº (%)	2 (16.7)	10 (83.3)
	% of total	2	10
E7b	nº (%)	4 (23.5)	13 (76.5)
	% of total	4	13
E8a	nº (%)	12 (48)	**13 (52)**
	% of total	12	13
E8b	nº (%)	0 (0)	**8 (100)**
	% of total	0	8
E20	nº (%)	0 (0)	4 (100)
	% of total	0	4
Minority profiles	nº (%)	17(60.7)	11 (39.3)
	% of total	17	11
TOTAL	nº (%)	39 (39)	61 (61)
	% total	39	61

In bold, ratios significant by pairwise comparison with test E. Fisher.

statistically significant (vancomycin, p = 0.004; tigecycline, p = 0.010). We subsequently performed the post-hoc Tukey's test, with the following significant results (table 3): a) No major genotype has an MIC of vancomycin or tigecycline that is significantly higher than that of any of the other major genotypes. b) The mean MIC value of tigecycline in isolates with the E7b pulsotype is significantly higher than that of isolates with the E10 pulsotype (p = 0.008). c) The mean MIC of vancomycin in isolates with the E8b pulsotype is significantly higher than that of isolates with the E8a (p = 0.018) and E10 (p = 0.030) pulsotypes.

We calculated the proportion of RVS isolates within each major genotype (table 5), and used Fisher's exact test to identify differences in these ratios between the major pulsotypes. The results indicated that these differences are significant (p = 0.002). The proportion of isolates with the E8b genetic profile that are characterized as RVS is significantly different from that of isolates with the E10 (p = 0.015) and E8a (p = 0.030) profiles, as indicated by the post-hoc pairwise comparison using Fisher's exact test (table 5). However, using the FDR method, no significant differences in the proportion of RVS isolates were obtained between pulsotypes.

To evaluate differences in the MICs values for linezolid and daptomycin between the major genotypic profiles, we used the Kruskal-Wallis test. The results indicated that these differences are statistically significant for linezolid (p = 0.034) but not for daptomycin (p = 0.96). To further analyze differences in linezolid resistance, pairwise comparisons were performed using the FDR method. The results indicated that the mean MIC value for linezolid in isolates with the E20 pulsotype is significantly lower than that observed in isolates with the E7a (p = 0.05), E7b (p = 0.038), and E8b (p = 0.038) profiles (table 3).

DISCUSSION

An analysis of the statistical results obtained using the FDR method revealed no significant differences in the resistance ratios exhibited by different genotypes for any of the antibiotics tested using the Vitek® system. However, the results obtained using Fisher's exact method indicated that the E8a and E10 genotypes exhibit higher resistance rates for levofloxacin than the other major pulsotypes. Similar results were obtained for the E[20] pulsotype with respect to mupirocin resistance. Genotype-associated susceptibility of MRSA isolates to gentamicin, tobramycin, erythromycin, clindamycin, fosfomycin, fusidic acid, and rifampin was not detected. We failed to detect resistance to the four antibiotics that are (i.e., vancomycin, tigecycline, daptomycin, and linezolid). However, because of the importance of antimicrobial sensitivity surveillance (i.e., monitoring increases in MIC) to therapeutic efficacy[16,17,22,23] it may be helpful to understand the associations between a particular genotype and reduced susceptibility to these antibiotics, which are commonly used in the treatment of MRSA in outbreaks of hospital infections and in severe community infections[24-26]. For vancomycin, tigecycline and linezolid, we found significant differences between the mean MIC values of the major genotypes. Isolates with the E7b genotype exhibit a significantly elevated MIC of tigecycline, isolates with the E8b genotype exhibit a significantly elevated MIC of vancomycin, and isolates with the E20 genotype exhibit a significantly reduced MIC of linezolid.

It is worth noting that none of the major genotypes had an MIC for any of the that was significantly higher than that of all the other major genotypes. Thus, the available data is not sufficient to establish priority antibiotics for MRSA treatment in a particular hospital or Health Area beyond the therapeutic indications attributed to each antibiotic according to patient clinical presentation (i.e., vancomycin in patients with bacteremia, linezolid in patients with pneumonia, tigecycline in patients with skin and soft tissue infections, and daptomycin in patients with bacteremia and endocarditis[27]). However, because the data obtained in this study indicate that isolates with certain pulsotypes exhibit higher MICs to some of these antibiotics, surveillance of antibiotic susceptibility in MRSA strains is important to prevent future resistance[28, 29]. Consistent with these conclusions, isolates with the E8b genotype exhibit a significantly elevated rate of RVS strains. In this case, 100% of the isolates have a vancomycin MIC of > 1mg/L. Because this reduced susceptibility is associated with treatment failure[16,17,30], we do not recommend treating a MRSA outbreak caused by this pulsotype with vancomycin. The same conclu-

sion can be made for isolates with the E20 pulsotype, of which 100% were also characterized as RVS. However, the low number of isolates of this genotype (i.e., only four isolates) makes this phenomenon a clear trend, which should be confirmed with a larger size sample.

We observed the presence of two MRSA genotypes in Extremadura, E17 and ESP, which are very infrequent (i.e., each observed in a single isolate) but highlight the existence of strains that exhibit antibiotic multiresistance. These isolates are resistant to gentamicin, tobramycin, levofloxacin, erythromycin, clindamycin, fosfomycin, and mupirocin (table 2) and exhibit reduced susceptibility to vancomycin and the highest recorded MIC of linezolid in the MRSA population studied (i.e., 2 mg/L) (table 4). These isolates have similar band profiles in PFGE, with only slight differences to distinguish them as different pulsotypes, and they have identical antibiotic resistance patterns. It is possible that the ESP isolate is a phylogenetic derivative of the E17 genotype. Although no previous studies have directly associated the E17 genotype with multidrug resistance and the number of isolates described in our study is small, the results reported here should be considered for future MRSA outbreaks.

In conclusion, although the correlations between MRSA genotypes and antibiotic susceptibility were not significant in post-hoc studies, the observed tendencies should be analysed in futures studies with larger sample sizes.

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