604
IDSA 2011
JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370, fax 319.665.3371
rodrigo-mendes@jmilabs.com

Evaluation of Occurrence and Resistance Mechanisms to Pleuromutilins in *Staphylococcus aureus* Clinical Isolates: Report from the SENTRY Antimicrobial Surveillance Program

RE MENDES, A COSTELLO, LM DESHPANDE, M CASTANHEIRA, RN JONES
JMI Laboratories, North Liberty, Iowa, USA

SENTRY ANTIMICROBIAL SURVEILLANCE

AMENDED ABSTRACT

Background: Topical antimicrobial agents for treating skin infections have been limited to fusidic acid and mupirocin. Recently, new options (pleuromutilins) have been made clinically available or are in development. This study aimed to evaluate the resistance mechanisms of *S. aureus* displaying elevated (≥4 µg/mL) tiamulin (pleuromutilins marker) MIC results during the 2010 SENTRY Antimicrobial Surveillance Program.

Methods: 10,025 *S. aureus* recovered from USA and European hospitals during the 2010 SENTRY Program were processed using CLSI methods (M100-S21, 2011). Seven strains showed elevated non-wildtype tiamulin MIC results (≥4 µg/mL). These strains were screened for mutations in the 23S rRNA, L3 and L4, and presence of *cfr* and *vga*(A-E) genes using PCR and sequencing. Clonality was examined via PVL genes, SCC*mec*, *spa* and MLST typing.

Results: Selected *S. aureus* isolates were mainly from Europe (57%; two countries) and all were methicillin-resistant. Two strains possessed a *vga*(A) variant and these *S. aureus* isolates were from the USA or France. Two *S. aureus* strains from the USA and one isolate from France harbored the *cfr* gene. Two strains from the Netherlands harbored *vga*(E). Mutations in the 23S rRNA and ribosomal proteins (L3/L4) were not observed. The *S. aureus* isolates belonged to ST398, ST88, ST8 or ST5.

Conclusions: Decreased susceptibility to tiamulin among *S. aureus* was rare (0.07%) and this result can be also inferred for other available pleuromutilins or those in clinical development. Evaluated isolates demonstrated decreased susceptibility mediated by the presence of an efflux pump (*vga*)-encoding gene or *cfr*. Target site alterations (mutations) were not detected in the studied strains, which were mostly associated to commonly detected lineages of *S. aureus* (ST5 and ST8) or ST398.

INTRODUCTION

Oral or topical antimicrobial therapy can be utilized for treating uncomplicated superficial skin infections caused by methicillin-resistant and susceptible *Staphylococcus aureus* as well as *Streptococcus pyogenes*. Topical agents could be preferred as administration can be directly targeted to the infected area, reducing the potential for systemic side-effects, such as gastrointestinal distress and undesired drug interactions. Until recently, topical antimicrobials have been limited to agents such as fusidic acid and mupirocin introduced in 1962 and 1985, respectively.

Recently, antimicrobial agents belonging to the pleuromutilin class have been approved or are in development for clinical use for treatment of uncomplicated skin and skin structure infections. These agents are protein synthesis inhibitors that target the large subunits of bacterial ribosomes. More specifically, they inhibit the peptide peptidyl transfer, block ribosomal P-site interactions, and inhibit normal ribosomal 50S subunit formation. Pleuromutilins demonstrate potent *in vitro* activity against Gram-positive and some Gram-negative organisms. Other in-class compounds, such as tiamulin and valnemulin have long been used in veterinary medicine to treat infections caused by Gram-positive pathogens.

While no relationship has been established between *in vitro* susceptibility of topical pleuromutilin agents and clinical efficacy, monitoring of *in vitro* activity is recommended for epidemiological purposes. In this study, we evaluated the resistance mechanisms and molecularly characterized seven *S. aureus* exhibiting elevated tiamulin MIC values at ≥4 µg/mL.

MATERIALS AND METHODS

Bacterial strain collection. A total of 10,025 *S. aureus* were recovered from geographically diverse medical centers in Europe and the USA during 2010 SENTRY Antimicrobial Surveillance Program platform. These clinical isolates were collected and submitted to a central monitoring laboratory (JMI Laboratories, Iowa, USA) following established protocols as part of the SENTRY Program. Seven (0.07%) *S. aureus* clinical isolates exhibited elevated non-wildtype tiamulin MIC results (≥4 µg/mL) and were selected for further analysis.

Antimicrobial susceptibility testing. Isolates were tested for susceptibility by broth microdilution method using validated dry-form panels (Thermo Fisher Scientific, Ohio, USA), according to Clinical and Laboratory Standards Institute (CLSI) methods (M07-A8, 2009). MIC results for selected strains were confirmed by using reference frozen-form panels. Validation of the minimum inhibitory concentration (MIC) values was performed by concurrent testing of CLSI-recommended (M100-S21, 2011) quality control (QC) strains: *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. Interpretation of MIC results was in accordance with published CLSI (M100-S21) breakpoint criteria, when available.

Screening for ribosomal target site mutations and resistance determinants. The 23S rRNA-, and L3- and L4-encoding genes were PCR-amplified and sequenced on both strands using the primers described in Table 1. Nucleotide and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Wisconsin, USA). Amino acid sequences were compared with those of *S. aureus* NCTC 8325. Isolates were screened for *vga*(A), *vga*(B), *vga*(C), *vga*(D) and *vga*(E) genes by standard PCR reactions. Strains previously characterized were used as positive controls and positive PCR products were confirmed by sequencing. Among selected strains, those displaying elevated linezolid MIC results (≥4 µg/mL) were also screened for *cfr*.

Molecular typing. The seven *S. aureus* isolates were further characterized by SCC*mec*, *spa* and multilocus sequence typing (MLST). *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/). MLST alleles and sequence types (ST) were identified using the MLST database (http://www.mlst.net) and the eBURST program (http://eburst.mlst.net) was utilized to infer the evolutionary relatedness among STs.

RESULTS

- Among 10,025 *S. aureus* clinical isolates recovered from the 2010 SENTRY Program sampling year, only seven (0.07%) exhibited elevated tiamulin MIC values (≥32 µg/mL). These isolates originated from France (two sites), Netherlands (one site) and the USA (three sites in three states; Table 2).
- The vast majority of studied strains (85.7%; 6/7) were recovered from male subjects between the ages of 34 and 56 (Table 2).
- All selected *S. aureus* were methicillin (oxacillin)-resistant, while variable susceptibility results were observed for other antimicrobial agents tested (Table 3). All strains were susceptible to linezolid (three strains with MIC of 4 µg/mL; upper limit of wildtype [WT] range), teicoplanin, vancomycin and trimethoprim-sulfamethoxazole.
- Two and one MRSA strains from the USA and France, respectively, harbored the *cfr* gene. These strains exhibited a $PhLOPS_A$ phenotype (i.e. resistance to phenicols, lincosamides, oxazolidinones [see above], pleuromutilins, and streptogramin A compounds; Table 3).
- Strains 27491 and 45573 were PCR-positive for *vga*(A), while *S. aureus* 44404 and 44410 harbored *vga*(E). These strains displayed elevated MIC results for tiamulin, clindamycin and quinupristin/dalfopristin, except for strain 27491 (Table 3).
- All selected *S. aureus* clinical isolates showed WT amino acid sequences for 23S rRNA, and the L3 and L4 ribosomal proteins (Table 3).
- Strains (two) originating from the Netherlands and France were *spa* t034 (ST398) and t008 (ST8), respectively. USA MRSA isolates were t008 (ST8), t242 (ST5) or t690 (ST88; Tables 2 and 3).

Table 1. Oligonucleotides used for PCR and sequencing.

Gene	Oligonucleotide	Sequence (5'-3')	Product size (bp)
23S rRNA	SA 23S-F	GCG GTC GCC TCC TAA AAG	420
	SA 23S-R	ATC CCG GTC CTC TCG TAC TA	
rplC	rplC-F	ACC CTG ATT TAG TTC CGT CTA	822
	rplC-R	GTT GAC GCT TTA ATG GGC TTA	
rplD	rplD-F	TCG CTT ACC TCC TTA ATG	1,200
	rplD-R	GGT AAC ACT GTA ACT G	
vga(A)	vga(A)-F	AGT GGT GGT GAA GTA ACA CG	1,287
	vga(A)-R	CTC TTG TTC TAA TTC TTC CG	
vga(A)v	vga(A)v-F	GAT TAT ACT CCG TGT TGA AGA TG	166
	vga(A)v-R	AAT ACC TGG CTT ATC CTG AAC	
vga(B)	vga(B)-F	CGA CAG TAT GAG TGG TGG TG	1,031
	vga(B)-R	CCT CAA CAG CAT CGA TAT CC	
vga(C)	vga(C)-F	CCG GAT TGG CTT GGT AGG TG	1,171
	vga(C)-R	GAG TGC TTC CGT ATC CAA GT	
vga(D)[a]	vga(D)-F	CAA CTG GAG CGA GCT GTT A	201
	vga(D)-R	GAC AGC CGG ATA ATC TTT TG	
vga(E)	vga(E)-F	AAT GGC ACG GGT AAA ACA AC	400
	vga(E)-R	TCG GTC GTG AGT AAC AAA TAG AAT	

a. Primers described in Jung *et al*., 2010.

Table 2. Demographic information associated with the *S. aureus* clinical isolates selected for this study.

Isolate	Country	State (USA)	City	Specimen	Age	Sex
22631	USA	Michigan	Lansing	Blood	34	M
22689	USA	Utah	Salt Lake City	Blood	55	M
27491	USA	North Dakota	Fargo	upper respiratory tract	66	F
52204	France	–	Metz	Blood	50	M
45573	France	–	Bron Cedex	Blood	56	M
44404	Netherlands	–	Nijmegen	Ears, nose or throat	37	M
44410	Netherlands	–	Nijmegen	Ears, nose or throat	44	M

Table 3. Antimicrobial susceptibility profiles and molecular characteristics of *S. aureus* selected for this study.

	Resistance mechanisms			Epidemiology				MIC (µg/mL) [susceptibility category][c]												
Isolate	vga	cfr	Mutations[a]	SCC*mecA*	*spa*	MLST	CC	TIA	OXA	CIP	CHL	CLI	ERY	GEN	LZD	Q/D	TET	TEC	VAN	SXT
22631	–	+	WT	IV	t008	ST8	8	>64	>2 [R]	>4 [R]	>128 [R]	>64 [R]	>4 [R]	≤1 [S]	4 [S]	2 [I]	≤0.12 [S]	≤1 [S]	0.5 [S]	≤0.5 [S]
22689	–	+	WT	II	t242	ST5	5	>64	>2 [R]	>4 [R]	32 [R]	>64 [R]	>4 [R]	>8 [R]	4 [S]	2 [I]	≤0.12 [S]	≤1 [S]	1 [S]	≤0.5 [S]
52204	–	+	WT	IV	t008	ST8	8	>64	>2 [R]	>4 [R]	>128 [R]	>64 [R]	≤0.25	≤1 [S]	4 [S]	2 [I]	≤0.12 [S]	≤1 [S]	1 [S]	≤0.5 [S]
27491	vgaA	–	WT	IV	t690	ST88	88	32	>2 [R]	0.25 [S]	8 [S]	≤0.25	0.5 [S]	≤1 [S]	1 [S]	1 [S]	0.25 [S]	≤1 [S]	1 [S]	1 [S]
45573	vgaA	–	WT	IV	t008	ST8	8	>64	>2 [R]	>4 [R]	8 [S]	>64 [R]	>4 [R]	≤1 [S]	1 [S]	2 [I]	0.25 [S]	≤1 [S]	1 [S]	≤0.5 [S]
44404	vgaE	–	WT	NT[b]	t034	ST398	398	>64	>2 [R]	0.25 [S]	8 [S]	>64 [R]	>4 [R]	≤1 [S]	1 [S]	2 [I]	>16 [R]	≤1 [S]	0.5 [S]	≤0.5 [S]
44410	vgaE	–	WT	NT	t034	ST398	398	>64	>2 [R]	1 [S]	8 [S]	>64 [R]	>4 [R]	≤1 [S]	1 [S]	4 [R]	>16 [R]	≤1 [S]	1 [S]	≤0.5 [S]

a. Mutations in 23S rRNA, L3 and L4 amino acid proteins. WT, wildtype.
b. NT, not typeable.
c. MIC interpretive criteria as published by CLSI M100-S21 (2011), when available. S, susceptible; I, intermediate; and R, resistant. TIA, tiamulin; OXA, oxacillin; CIP, ciprofloxacin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; Q/D; quinupristin/ dalfopristin; TET, tetracycline; TEC, teicoplanin; VAN, vancomycin; and SXT, trimethoprim/sulfamethoxazole.

CONCLUSIONS

- Overall, this study reports a very low rate (7/10,025; 0.07%) for decreased susceptibility to tiamulin (MIC values, ≥4 µg/mL; range 32 – >64 µg/mL) among *S. aureus* clinical isolates in the USA and Europe. These results can also be inferred for other available pleuromutilins or those in clinical development.
- The pleuromutilin resistance mechanisms observed were mostly associated with the presence of *cfr* or *vga* genes and not due to mutations in the 23S rRNA or ribosomal proteins. These results are in agreement with the fact that mutations are more likely to occur during prolonged selective pressure.
- The selected strains belonged to several different clonal complexes (CC), suggesting a very diverse genetic background.

ACKNOWLEDGEMENTS

The authors would like to thank the SENTRY Antimicrobial Surveillance Program participating medical sites that contributed *S. aureus* clinical isolates for this study: A. Croft (Salt Lake City, Utah), J. Etienne (Bron Cedex, France), W.T. Khalife (Lansing, Michigan), M. Loock (Fargo, North Dakota), J.W. Mouton (Nijmegen, The Netherlands) and Y. Rio (Metz, France).

SELECTED REFERENCES

1. Clinical and Laboratory Standards Institute (2009). *M07-A8. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard: eighth edition.* Wayne, PA: CLSI.
2. Clinical and Laboratory Standards Institute (2011). *M100-S21. Performance standards for antimicrobial susceptibility testing: 21st informational supplement.* Wayne, PA: CLSI.
3. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 38: 1008-1015.
4. Gentry DR, McCloskey L, Gwynn MN, Rittenhouse SF, Scangarella N, Shawar R, Holmes DJ (2008). Genetic characterization of Vga ABC proteins conferring reduced susceptibility to pleuromutilins in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 52: 4507-4509.
5. Gentry DR, Rittenhouse SF, McCloskey L, Holmes DJ (2007). Stepwise exposure of *Staphylococcus aureus* to pleuromutilins is associated with stepwise acquisition of mutations in *rplC* and minimally affects susceptibility to retapamulin. *Antimicrob Agents Chemother* 51: 2048-2052.
6. Jung YH, Shin ES, Kim O, Yoo JS, Lee KM, Yoo JI, Chung GT, Lee YS (2010). Characterization of two newly identified genes, *vga*(D) and *vat*(H), conferring resistance to streptogramin A in *Enterococcus faecium*. Antimicrob Agents Chemother 54: 4744-4749.
7. Kosowska-Shick K, Clark C, Credito K, McGhee P, Dewasse B, Bogdanovich T, Appelbaum PC (2006). Single- and multistep resistance selection studies on the activity of retapamulin compared to other agents against *Staphylococcus aureus* and *Streptococcus pyogenes*. *Antimicrob Agents Chemother* 50: 765-769.
8. Milheirico C, Oliveira DC, de Lencastre H (2007). Update to the multiplex PCR strategy for assignment of mec element types in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 51: 3374-3377.
9. Schwendener S and Perreten V (2011). New transposon Tn6133 in methicillin-resistant *Staphylococcus aureus* ST398 contains *vga*(E), a novel streptogramin A, pleuromutilin, and lincosamide resistance gene. Antimicrob. Agents Chemother 55: 4900-4904.
10. Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN (1999). Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J Clin Microbiol* 37: 3556-3563.