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Rare Occurrence of Retapamulin Resistance Mechanisms in *Staphylococcus aureus* Clinical Isolates: Report from the SENTRY Program

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SENTRY ANTIMICROBIAL SURVEILLANCE

Abstract

Background: Retapamulin has been approved (2007) for impetigo treatment in USA and uncomplicated skin infections and impetigo in Europe. Decreased susceptibility to retapamulin has been associated with *vga*(A) variant (v) and mutations in the L3 ribosomal protein. We evaluated the resistance mechanisms and molecular characteristics of eleven *S. aureus* exhibiting retapamulin MIC values of ≥2 μg/mL.

Methods: 10,640 *S. aureus* from the SENTRY Program (2008) were processed using CLSI methods. Eleven (0.1%) strains showed elevated non-wildtype retapamulin MIC results (2 – 8 μg/mL) and were screened for L3 and L4 mutations, and presence of *vga* genes using PCR/sequencing. Clonality was examined via PVL genes and SCC*mec*, *agr*, PFGE, *spa* and MLST typing. Gene location was assessed by Southern blot/hybridization and PCR using a primer anchored to *att554*.

Results: Isolates were commonly (82%) from France (4 cities). All *S. aureus* had *vga*(A)v and one isolate also harbored *vga*(B). All strains were wildtype for L3 and L4 and PVL-negative. Six (54.5%) *S. aureus* possessed SCC*mec* III or IV (MRSA). Four strains were related by PFGE. Most isolates (82%) were MLST-8, *agr* 1, t008 or 1 or 2 allelic variants of t008 (probably the Lyon clone), and the remaining two were MLST-5, *agr* 2, t002 and MLST-239, *agr* 1, t037 (Hungarian clone). *vga*(A)v was chromosomally located in all *S. aureus* and adjacent to *att554* in nine strains.

Isolate No.	Country	City	*vga*(A)v	*vga*(B)	SCC*mec*	*agr*	*spa*	MLST	PFGE pattern	RET MIC (μg/mL)
1194	France	Metz	+	-	NA[a]	1	t008	8	B	2
1311	France	Caen	+	-	NA	1	t024	8	E	2
2183	France	Lille	+	-	NA	1	t648	8	A2	4
3862	USA	Omaha	+	-	NA	2	t002	5	H	4
9681	Switzerland	Lausanne	+	-	NA	1	t008	8	G	4
1564	France	Lille	+	+	IV	1	t2054	8	D	4
5087	France	Bron	+	-	IV	1	t008	8	A	2
5092	France	Bron	+	-	IV	1	t008	8	A	2
2643	France	Bron	+	-	IV	1	t008	8	A1	8
4370	France	Caen	+	-	IV	1	t1171	8	F	2
1203	France	Metz	+	-	III	1	t037	239	C	4

a. NA = Not applicable (*mec*A-negative)

Conclusions: Decreased susceptibility to retapamulin was rare (0.1%), dominantly in MLST-8 lineage strains from France and associated with *vga*(A)v. The chromosomal location of *vga*(A)v may limit the mobility of this resistance determinant.

Introduction

Antimicrobial agents belonging to the pleuromutilin class are protein synthesis inhibitors that target the large subunits of bacterial ribosomes. More specifically, these agents inhibit peptide peptidyl transfer, block ribosomal P-site interactions, and inhibit normal ribosomal 50S subunit formation in the ribosomes. Pleuromutilins demonstrate potent *in vitro* activity against Gram-positive and some -negative organisms, and in-class compounds such as tiamulin and valnemulin, are currently used in veterinary medicine to treat infections caused by Gram-positive pathogens.

Retapamulin ointment 1% (Altabax®/Altargo®, GlaxoSmithKline) was the first pleuromutilin compound approved for clinical use in humans for treatment of uncomplicated superficial skin infections caused by methicillin-susceptible *Staphylococcus aureus* and *Streptococcus pyogenes*. This drug was approved in 2007 for treating impetigo or small infected wounds in Europe, and for impetigo in the United States (USA).

Decreased susceptibility to pleuromutilin compounds has been associated with efflux-pumps [e.g. *vga*(A) variant (v)], mutations in the L3 and L4 ribosomal proteins and *cfr*. In this study, we evaluated the resistance mechanisms and molecularly characterized eleven *S. aureus* exhibiting retapamulin MIC values at ≥2 μg/mL. While no relationship has been established between *in vitro* susceptibility of retapamulin and clinical efficacy, monitoring of *in vitro* activity is recommended for epidemiological purposes.

Methods

Bacterial strain collection. A total of 10,640 *S. aureus* were recovered from geographically distributed medical centers in the Americas, Europe and the Asia-Pacific region during 2008. These clinical isolates were collected and submitted to a central monitoring laboratory (JMI Laboratories, IA) as part of the SENTRY Antimicrobial Surveillance Program, following established protocols. Eleven *S. aureus* (0.1%) exhibited elevated non-wildtype retapamulin MIC results (≥2 μg/mL) and were selected for further analysis.

Antimicrobial susceptibility testing. Isolates were tested for susceptibility by reference broth microdilution method using cation-adjusted Mueller-Hinton broth in GMP prepared and validated panels (TREK Diagnostics Inc., OH), according to Clinical and Laboratory Standards Institute (CLSI) methods (M07-A8, 2009). Validation of the minimum inhibitory concentration (MIC) values was performed by concurrent testing of CLSI-recommended (M100-S20-U, 2010) quality control (QC) strains: *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. Interpretation of MIC results was in accordance with published CLSI (M100-S20-U) breakpoint criteria.

Screening for ribosomal target site mutations and resistance determinants. The L3- and L4-encoding genes were PCR-amplified and sequenced on both strands using primers described in Table 1. Nucleotide and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, WI). Amino acid sequences were compared with those of *S. aureus* NCTC 8325. Isolates were screened for *vga*(A) and *vga*(B) genes by standard PCR reactions using primers listed in Table 1. Strains previously characterized were used as positive controls and positive PCR products were confirmed by sequencing.

Molecular typing. Pulsed-field gel electrophoresis (PFGE) of SmaI digests was performed to investigate clonality and pattern analysis carried out using the GelCompar II software (Applied Math, Kortrijk, Belgium). Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 0.9% and 0.5%, respectively. Isolates showing similarity coefficient ≥80% were considered as genetically related. *S. aureus* were further characterized by SCC*mec*, *spa*, *agr* and multilocus sequence typing (MLST).

Plasmid analysis and *vga*(A) location. Plasmid DNA was extracted using the Plasmid DNA Mini Kit (Qiagen GmbH, Hilden, Germany) and separated on 1% agarose gel. Plasmid DNA bands were transferred onto a nylon membrane by Southern blot. *cfr*-specific labeled probe was generated and membrane hybridized using the nonradioactive DIG High Prime DNA labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). As the *vga*(A) gene was previously associated with Tn*5406*, which preferentially inserts into the chromosomal locus *att554*, the gene location was PCR-mapped using primers anchoring to *att554* and *vga*(A).

Results

- Among 10,640 *S. aureus* clinical isolates recovered from the 2008 SENTRY sampling year, 11 (0.1%) exhibited elevated retapamulin MIC values (range, 2 – 8 μg/mL). All of these 11 isolates originated from France (four cities), except for one strain each from the USA and Switzerland (Table 2).
- Overall, the selected *S. aureus* were susceptible to chloramphenicol, linezolid, tetracycline, trimethoprim/sulfamethoxazole and glycopeptides. Variable susceptibility results were noted for clindamycin, gentamicin, erythromycin, ciprofloxacin and oxacillin (Table 2).
- All of the 11 *S. aureus* were PCR-positive for *vga*(A)v and the encoded protein showed highest identity with that under the accession number AF186237. One isolate also harbored *vga*(B) and all strains were wildtype for L3 and L4, and PVL-negative. Among oxacillin-resistant strains (six; 54.5%), SCC*mec* type III or IV were detected (Figure 1).
- Nine (82%) of the 11 isolates were sequence type (ST)-8, *agr* 1, t008 or 1 or 2 allelic variants of t008 (t648, t2054, t1171 and t024), and the remaining two strains were ST-5, *agr* 2, t002 and MLST-239, *agr* 1, t037 (Figure 1).
- Except for strains 1203 and 3862, the remaining selected *S. aureus* isolates originated from France or a geographically close region of Switzerland and were associated with ST-8. In addition, these isolates belonging to ST-8 lineages clustered within six PFGE groups.
- The *vga*(A)v gene was chromosomally located in all *S. aureus* and PCR-mapping demonstrated that this gene was adjacent to *att554* in nine strains.

Table 1. Oligonucleotides used for PCR and sequencing.

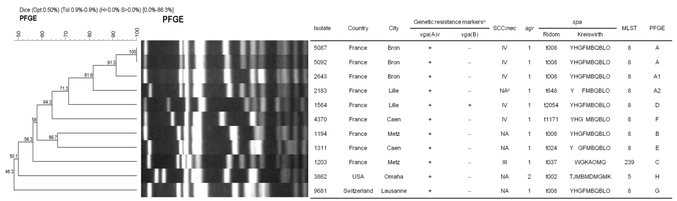
Gene	Oligonucleotide	Sequence (5'-3')	Product size (bp)
rplC	rplC-F	ACC CTG ATT TAG TTC CGT CTA	822
	rplC-R	GTT GAC GCT TTA ATG GGC TTA	
rplD	rplD-F	TCG CTT ACC TCC TTA ATG	1200
	rplD-R	GGT AAC ACT GTA ACT G	
vga(A)	vga(A)-F	AGT GGT GGT GAA GTA ACA CG	1287
	vga(A)-R	CTC TTG TTC TAA TTC TTC CG	
vga(B)	vga(B)-F	CGA CAG TAT GAG TGG TGG TG	1031
	vga(B)-R	CCT CAA CAG CAT CGA TAT CC	

Table 2. Antimicrobial susceptibility profile of *vga*(A)-carrying *S. aureus* selected for this study.

			MIC (μg/mL) [susceptibility category][a]												
Isolate	Specimen Type	Country	RET	OXA	CIP	CHL	CLI	ERY	GEN	LZD	Q/D	TET	TEC	VAN	SXT
5087	Bone/Joint	France	2	>2 [R]	>4 [R]	4 [S]	0.5 [S]	0.25 [S]	≤2 [S]	2 [S]	1 [S]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
5092	Blood	France	2	>2 [R]	>4 [R]	4 [S]	0.5 [S]	0.25 [S]	≤2 [S]	2 [S]	1 [S]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
2643	Bone/Joint	France	8	>2 [R]	>4 [R]	4 [S]	0.5 [S]	8 [R]	≤2 [S]	1 [S]	>2 [R]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
2183	Tracheal aspirate	France	4	0.5 [S]	>4 [R]	4 [S]	0.25 [S]	0.25 [S]	≤2 [S]	2 [S]	2 [I]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
1564	Abscess (pus)	France	4	>2 [R]	>4 [R]	4 [S]	>256 [R]	>256 [R]	>8 [R]	1 [S]	>2 [R]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
4370	Blood	France	2	>2 [R]	>4 [R]	4 [S]	0.25 [S]	0.25 [S]	≤2 [S]	1 [S]	0.5 [S]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
1194	Skin/Soft Tissue	France	2	0.5 [S]	>4 [R]	4 [S]	0.25 [S]	>256 [R]	≤2 [S]	1 [S]	1 [S]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
1311	Wound/Drainage/Ulcer	France	2	0.5 [S]	0.5 [S]	2 [S]	0.5 [S]	0.12 [S]	≤2 [S]	2 [S]	0.5 [S]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
1203	Skin/Soft Tissue	France	4	>2 [R]	>4 [R]	2 [S]	0.5 [S]	0.5 [S]	>8 [R]	2 [S]	1 [S]	>8 [R]	≤2 [S]	1 [S]	≤0.5 [S]
3862	Blood	USA	4	0.5 [S]	0.5 [S]	2 [S]	0.25 [S]	0.25 [S]	≤2 [S]	2 [S]	1 [S]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]
9681	Blood	Switzerland	4	0.5 [S]	0.5 [S]	4 [S]	0.5 [S]	0.25 [S]	≤2 [S]	2 [S]	1 [S]	≤2 [S]	≤2 [S]	1 [S]	≤0.5 [S]

a. MIC interpretive criteria as published by CLSI M100-S20-U, when available. S, susceptible; I, intermediate; and R, resistant. RET, retapamulin; 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.

Figure 1. Genetic resistance determinants, SCC*mec* type, *agr* allele, *spa* and MLST typing results and PFGE pattern analysis of selected *S. aureus* included in this study. Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 0.9% and 0.5%, respectively. Isolates showing similarity coefficient ≥80% were considered as genetically related. a. All tested isolates showed wildtype L3 and L4 ribosomal proteins. b. NA, reads not applicable.



Isolate	Country	City	Genetic resistance markers[a]		SCC*mec*	*agr*	*spa*		MLST	PFGE
			vga(A)v	*vga*(B)			Ridom	Kreiswirth		
5087	France	Bron	+	–	IV	1	t008	YHGFMBGBLO	8	A
5092	France	Bron	+	–	IV	1	t008	YHGFMBGBLO	8	A
2643	France	Bron	+	–	IV	1	t008	YHGFMBGBLO	8	A1
2183	France	Lille	+	–	NA[b]	1	t648	Y FMBGBLO	8	A2
1564	France	Lille	+	+	IV	1	t2054	YHGFMBGBLO	8	D
4370	France	Caen	+	–	IV	1	t1171	YHG MBGBLO	8	F
1194	France	Metz	+	–	NA	1	t008	YHGFMBGBLO	8	B
1311	France	Caen	+	–	NA	1	t024	Y GFMBGBLO	8	E
1203	France	Metz	+	–	III	1	t037	WGKAOMQ	239	C
3862	USA	Omaha	+	–	NA	2	t002	TJMBMDMGMK	5	H
9681	Switzerland	Lausanne	+	–	NA	1	t008	YHGFMBGBLO	8	G

Conclusions

- This study reports a very low rate (11/10,640; 0.1%) for decreased susceptibility to retapamulin (MIC values, ≥2 μg/mL) among a global collection of *S. aureus* clinical isolates recovered during the 2008 SENTRY Program.
- The reduced retapamulin susceptibility observed among these *S. aureus* strains was conferred by the presence of *vga*(A)v and not associated with mutations in ribosomal proteins.
- The strains were geographically concentrated in French hospitals, associated with ST-8, *spa* t008 or derivatives, *agr* 1 allele, SCC*mec* type IV (among MRSA) and PVL-negative. These genetic characteristics are compatible with those from *S. aureus* belonging to the major hospital-associated Lyon clone, which predominates among French medical institutions.

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