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Resistance Mechanisms Associated with Pleuromutilins among Gram-Positive Clinical Isolates from the Worldwide Surveillance Program for Lefamulin in 2018

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INTRODUCTION

- Lefamulin (Xenleta™) was recently approved by the US Food and Drug Administration for the treatment of community acquired bacterial pneumonia (CABP).
  - Both intravenous and oral formulations are available.
  - Lefamulin is a first-in-class, semi-synthetic pleuromutilin that inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit at the A- and P-sites in the peptidyl transferase center (PTC) via an "induced-fit" mechanism, which prohibits the correct positioning of the tRNA.
- The *in vitro* activity of lefamulin and emergence of resistance have been monitored against a global collection of Gram-positive and fastidious Gram-negative organisms through the SENTRY Antimicrobial Surveillance Program.
- This study evaluated the resistance mechanisms associated with elevated lefamulin MIC values in a global collection of surveillance isolates collected during 2018.

MATERIALS AND METHODS

Bacterial isolates

- A total of 4,406 *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS), *Streptococcus pneumoniae* and β-haemolytic and viridans group streptococci were included as part of the surveillance study for 2018 (Table 1).
  - A total of 36 (0.8%) isolates met the MIC screening criteria based on the FDA breakpoints or tentative epidemiological cut-off (ECOFF) values:
    - *S. aureus*: ≥0.5 µg/mL (FDA susceptible breakpoint ≤0.25 µg/mL)
    - CoNS, β-haemolytic *Streptococcus* spp.: ≥0.5 µg/mL
    - *S. pneumoniae*: ≥1 µg/mL (FDA susceptible breakpoint ≤0.5 µg/mL)
    - *S. bovis* group and *S. salivarius* group: ≥1 µg/mL
- Bacterial isolate identification was confirmed by standard algorithms supported by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany) and genome sequencing.

Antimicrobial susceptibility testing

- Isolates were tested for susceptibility by broth microdilution following Clinical and Laboratory Standards Institute (CLSI) M07 (2018) guidelines.
- Frozen-form broth microdilution panels were manufactured by JMI Laboratories (North Liberty, Iowa) and contained cation-adjusted Mueller-Hinton broth (2.5–5% lysed horse blood added for testing streptococci).
- Quality assurance was performed by concurrently testing CLSI-recommended quality control reference strains (*S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *S. pneumoniae* ATCC 49619).

Characterization of resistance mechanisms by next-generation sequencing

- Selected isolates had total genomic DNA extracted by the fully automated Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor (Cleveland, OH, USA), which was used as input material for library construction.
- DNA libraries were prepared using the Nextera™ library construction protocol (Illumina, San Diego, CA, USA) following the manufacturer's instructions and were sequenced on MiSeq Sequencer platforms at JMI Laboratories.
- FASTQ format sequencing files for each sample set were assembled independently using *de novo* assembler SPAdes 3.9.0. An in-house software was applied to align the assembled sequences against known macrolide, lincosamide and streptogramin B (MLS_B) and pleuromutilin resistance genes.
- Additional sequences of intrinsic genes associated with pleuromutilin binding site, including 23S rRNA (peptidyl transferase center [PTC]), *rplC* (L3), *rplD* (L4) and *rplV* (L22) were evaluated against a susceptible reference strain of the corresponding species.
- All intrinsic 23S rRNA target genes or ribosomal protein amino acid sequences were considered wild type if 100.0% homology with the respective reference sequences was displayed.
  - Differences were annotated when <100.0% homology was observed.

Multilocus sequence typing

- Multilocus sequence typing (MLST) was performed by extracting the previously defined set of 7 housekeeping gene fragments (~500 bp).
  - Each fragment was compared to known allelic variants for each locus (housekeeping gene) on the MLST website (PubMLST, https://pubmlst.org).
- An allele sharing 100% genetic identity with a known variant received a numeric designation.
  - A 7-number sequence (1 for each housekeeping gene) formed an allelic profile, defined as sequence types (STs).
  - Isolates containing alleles that did not match an existing sequence in the MLST database were submitted/deposited for allele and ST assignments.

RESULTS

- Among 1,607 *S. aureus* included in the 2018 lefamulin surveillance program, 8 (0.5%) isolates had lefamulin MIC values of 1–>16 mg/L and were selected for this study (Table 1). All other *S. aureus* isolates showed lefamulin MICs of ≤0.008–0.25 mg/L ($MIC_{50/90}$ of 0.06/0.12 mg/L).
  - None of the *S. aureus* isolates (0%) harboured the methyl transferase encoding gene *cfr*.
  - All 8 *S. aureus* harboured *vga*(A) (6/8; lefamulin MIC, 1–8 mg/L) or *lsa*(E) (2/8; lefamulin MIC, >32 mg/L) (Table 2).
- A total of 24 (8.9%) CoNS with lefamulin MIC values of 0.5–>32 mg/L were observed during the surveillance study and selected for genetic characterization. All other CoNS demonstrated lefamulin MICs of ≤0.008–0.25 mg/L ($MIC_{50/90}$ of 0.06/0.25 mg/L).
  - 20 (7.4%) were selected for further characterization, as 3 *S. cohnii* and 1 *S. sciuri* were not included due to known intrinsic decreased susceptibility to lefamulin (Table 1).
  - CoNS carried either *vga* gene variants (18/20; lefamulin MIC, 2–>32 mg/L) or showed G2576T alterations in the 23S rRNA (Table 3).
  - 6 CoNS isolates also had L3 mutations at H146 and M156 or at position V154 (2/20; lefamulin MIC of 0.5 mg/L) (Table 3).
- Only 2 of 1,866 (0.1%) *S. pneumoniae* were non-susceptible to lefamulin according to FDA breakpoints and showed lefamulin MICs of 1–2 mg/L. All other isolates displayed lefamulin MICs of ≤0.008–0.5 mg/L ($MIC_{50/90}$ of 0.06/0.25 mg/L) (Table 1).
  - Both isolates had mutations in ribosomal proteins (L4 or in L3 and L22) (Table 4).
- The sole *S. oralis* isolate with an elevated lefamulin MIC value (1 mg/L) did not show any resistance mechanisms and this MIC might belong to the normal wildtype distribution (Table 4).
- Among other streptococci, 3 of 522 (0.6%) β-haemolytic and 2 of 141 (1.4%) viridans group streptococci carried *lsa*(E) (lefamulin MIC, 2–32 mg/L) (Table 4).

Table 2. Molecular epidemiology and resistance mechanism results for *S. aureus* isolates

Collection no.	MLST	Country	MIC[a] (µg/mL)	Resistance mechanisms						
				Gene			Ribosomal mutations[b]			
				cfr	*lsa*(E)	*vga*(A)	23S rRNA	L3	L4	L22
1068133	9	Costa Rica	1 (1)	-	-	+	WT	WT	WT	WT
1070725	8	Chile	2 (1)	-	-	+	WT	WT	WT	WT
1059235	8	France	2 (2)	-	-	+	WT	WT	WT	WT
1053686	8	Portugal	2 (2)	-	-	+	WT	WT	WT	WT
1058562	398	Italy	8 (4)	-	-	+	WT	WT	WT	WT
1061409	398	Italy	8 (4)	-	-	+	WT	WT	WT	WT
1056173	9	Mexico	>32 (>16)	-	+	-	WT	WT	WT	WT
1084259	398	Russia	>32 (>16)	-	+	-	WT	WT	WT	WT

MLST, multilocus sequence typing; WT, wild type
[a] MIC, minimal inhibitory concentration; initial lefamulin MIC results obtained during the surveillance studies are within parentheses
[b] 23S rRNA mutational analysis performed on nucleotide sequences. Mutations outside of PTC were observed, but were considered as polymorphisms and suppressed here and the 23S rRNA sequence designated as WT. Protein sequences analyzed for annotating L3, L4 and L22

Table 1. Lefamulin MIC distributions obtained during the surveillance program for 2018. Clinical isolates included in this study are in red font.

Organism/organism group (no. of isolates)	No. and cumulative % of isolates inhibited at MIC (mg/L) of:[a]													MIC_{50}	MIC_{90}
	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	>16		
Staphylococcus aureus (1,607)	1 0.1	3 0.2	89 5.8	1,118 75.4	368 98.3	20 99.5	0 99.5	2 99.6	2 99.8	2 99.9	0 99.9	0 99.9	2 100.0	0.06	0.12
Coagulase-negative staphylococci (270)	2 0.7	11 4.8	87 37.0	129 84.8	12 89.3	5 91.1	4 92.6	6 94.8	5 96.3	1 96.7	2 97.4	2 98.5	4 100.0	0.06	0.25
S. capitis (11)	0	1	2	7	0	0	0	0	0	0	0	1		0.06	0.06
S. cohnii (3)[b]						0	2	0	1					0.5	
S. epidermidis (158)	1	5	51	78	7	1	1	6	4	1	2			0.06	0.5
S. haemolyticus (27)		0	1	20	2	0	0	0	0	0	0	1	3	0.06	>16
S. hominis (27)		0	9	14	1	2	1							0.06	0.25
S. lugdunensis (28)	1	4	20	3										0.03	0.06
S. saprophyticus (5)			0	2	1	2									
S. warneri (6)	0	1	1	3	1									0.12	
S. sciuri (1)[c]													1		
Streptococcus pneumoniae (1,866)	1 0.1	23 1.3	169 10.3	743 50.2	700 87.7	224 99.7	4 99.9	1 99.9	1 100.0					0.06	0.25
β-hemolytic streptococci (522)	4 0.8	49 10.2	347 76.6	97 95.2	19 98.9	3 99.4	0 99.4	1 99.6	0 99.6	0 99.6	0 99.6	0 99.6	2 100.0	0.03	0.06
Streptococcus pyogenes (205)	3 1.5	44 22.9	126 84.4	30 99.0	1 99.5	1 100.0								0.03	0.06
Streptococcus agalactiae (235)	1 0.4	4 2.1	177 77.4	36 92.8	15 99.1	1 99.6	0 99.6	0 99.6	0 99.6	0 99.6	0 99.6	0 99.6	1 100.0	0.03	0.06
Streptococcus dysgalactiae (82)	0 0.0	1 1.2	44 54.9	31 92.7	3 96.3	1 97.6	0 97.6	1 98.8	0 98.8	0 98.8	0 98.8	0 98.8	1 100.0	0.03	0.06
Viridans group streptococci (141)	9 6.4	12 14.9	15 25.5	23 41.8	29 62.4	30 83.7	2 85.1	4 87.9	10 95.0	5 98.6	0 98.6	0 98.6	2 100.0	0.12	2
Streptococcus anginosus group (45)	4 8.9	6 22.2	5 33.3	7 48.9	9 68.9	14 100.0								0.12	0.25
Streptococcus bovis group (30)	1 3.3	5 20.0	4 33.3	0 33.3	0 33.3	2 40.0	0 40.0	2 46.7	9 76.7	5 93.3	0 93.3	0 93.3	2 100.0	2	4
Streptococcus mitis group (46)	1 2.2	0 2.2	4 10.9	8 28.3	15 60.9	13 89.1	2 93.5	2 97.8	1 100.0					0.12	0.5
Streptococcus salivarius group (20)	3 15.0	1 20.0	2 30.0	8 70.0	5 95.0	1 100.0								0.06	0.12

[a] Shaded MIC represent those above the breakpoints or ECOFF values for the respective species or groups of species
[b] *S. cohnii* is known to be less susceptible to lefamulin compared to other CoNS species and these isolates were not selected
[c] *S. sciuri* is known to carry *sal*(A) that causes pleuromutilin, lincosamide and streptogramin A resistance

Table 3. Molecular epidemiology and resistance mechanisms results for *Staphylococcus* spp. other than *S. aureus* included in this study

Collection no.	Organism	MLST	Country	MIC[a] (µg/mL)	Resistance mechanisms				
				Gene	Ribosomal mutations[b]				
				vga	23S rRNA	L3	L4	L22
1084078	*S. hominis*	2	Russia	1 (0.5)	-	G2576T	V154L, M156T	WT	WT
1076534	*S. epidermidis*	2	France	1 (0.5)	-	G2576T	H146P, M156T	WT	WT
1074181	*S. epidermidis*	87	Belgium	2 (1)	*vga*(A), *vga*(B)	WT	WT	WT	WT
1067361	*S. epidermidis*	87	Australia	2 (1)	*vga*(A), *vga*(B)	WT	WT	WT	WT
1060764	*S. epidermidis*	87	Italy	2 (1)	*vga*(A), *vga*(B)	WT	WT	WT	WT
1056376	*S. epidermidis*	87-like	France	2 (1)	*vga*(A), *vga*(B)	WT	WT	WT	WT
1067269	*S. epidermidis*	87	Australia	2 (2)	*vga*(A), *vga*(B)	WT	WT	WT	WT
1049306	*S. epidermidis*	57-like	USA	2 (2)	*vga*(A)	WT	WT	WT	WT
1054755	*S. epidermidis*	16	USA	4 (1)	*vga*(A)	WT	A142T	WT	WT
1083242	*S. epidermidis*	59	Taiwan	4 (2)	*vga*(A)	WT	WT	WT	WT
1042824	*S. epidermidis*	2-like	USA	4 (8)	*vga*(A)	WT	WT	WT	WT
1066841	*S. epidermidis*	73	USA	8 (1)	*vga*(A)	WT	WT	WT	WT
1066794	*S. epidermidis*	2	Australia	16 (2)	*vga*(A)	WT	WT	WT	WT
1059860	*S. epidermidis*	54	England	16 (4)	*vga*(A)	WT	WT	WT	WT
1043387	*S. epidermidis*	130	USA	16 (8)	*vga*(A)	WT	WT	WT	WT
1074687	*S. capitis*	NA	Korea	16 (16)	*vga*(A)	WT	T83A	WT	WT
1058385	*S. haemolyticus*	65	England	16 (16)	*vga*(A) LC	WT	WT	WT	WT
1068052	*S. haemolyticus*	55-like	Costa Rica	32 (>16)	*vga*(A) LC	WT	A82T, A137G	WT	WT
1081281	*S. haemolyticus*	3	Thailand	>32 (>16)	*vga*(A), *vga*(A) LC	WT	V154L, M156T	WT	WT
1053738	*S. haemolyticus*	29	Portugal	>32 (>16)	*vga*(A) LC	WT	WT	WT	WT

MLST, multilocus sequence typing; NA, MLST method and database non-existent; WT, wild type
[a] MIC, minimal inhibitory concentration; initial lefamulin MIC results obtained during the surveillance studies are within parentheses
[b] 23S rRNA mutational analysis performed on nucleotide sequences. Mutations outside of PTC were observed, but were considered as polymorphisms and suppressed here and 23S rRNA sequence designated as WT. Protein sequences analyzed for annotating L3, L4 and L22

Table 4. Molecular epidemiology results and resistance mechanisms obtained for *Streptococcus* spp. selected for this study

Collection no.	Organism	Country	MIC[a] (µg/mL)	Resistance mechanisms				
				Genes	Ribosomal mutations[b]			
					23S rRNA	L3	L4	L22
1077452	*S. pneumoniae*	Spain	1 (1)	-	WT	WT	S20N, A197V	WT
1047147	*S. pneumoniae*	USA	2 (2)	-	WT	P24S	WT	K20E
1075633	*S. oralis*	Sweden	1 (2)	-	WT	WT	WT	WT
1063698	*S. dysgalactiae*	Malaysia	2 (1)	*lsa*(E)	WT	WT	WT	WT
1047256	*S. dysgalactiae*	USA	32 (>16)	*lsa*(E)	WT	WT	K141N	WT
1069862	*S. agalactiae*	Mexico	16 (>16)	*lsa*(E)	WT	WT	WT	WT
1067265	*S. gallolyticus*	Australia	16 (>16)	*lsa*(E)	WT	E29K	WT	WT
1087306	*S. gallolyticus*	Italy	32 (>16)	*lsa*(E)	WT	WT	WT	WT

WT, wild type
[a] MIC, minimal inhibitory concentration; initial lefamulin MIC results obtained during the surveillance studies are within parentheses
[b] 23S rRNA mutational analysis performed on nucleotide sequences. Mutations outside of PTC were observed, but were considered as polymorphisms and suppressed here and sequence designated as WT. Protein sequences analyzed for annotating L3, L4 and L22

CONCLUSIONS

- Gram-positive isolates from a global collection causing human infections and exhibiting elevated lefamulin MICs were rare.
- Lefamulin resistance mechanisms identified in *S. aureus*, CoNS and streptococcal isolates mostly included *vga*(A) and *lsa*(E), which have been described to mediate ribosomal protection. *vga* genes remained more prevalent in staphylococci, whereas *lsa*(E) prevailed among streptococci.
- Target site alterations such as mutations in the large ribosomal proteins or 23S rRNA were less common, and the *cfr* gene was not detected among the Gram-positive surveillance isolates from this study.
- Longitudinal surveillance studies will continue to monitor for emergence of resistance and stability of the *in vitro* activity of lefamulin among gram-positive clinical isolates.

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