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Molecular Characterization of Resistance Mechanisms Associated With Pleuromutilins Among Gram-Positive Clinical Isolates From the Worldwide SENTRY Surveillance Studies for Lefamulin

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ABSTRACT

Background: Lefamulin (LEF) is the first pleuromutilin antibiotic under clinical development for IV and oral use in humans. LEF recently completed the first of 2 phase 3 clinical trials for the treatment of community-acquired bacterial pneumonia in adults. This study evaluated the resistance mechanisms associated with elevated LEF MICs in a global collection of surveillance isolates from 2015–2016.

Methods: A total of 3,195 staphylococci and 4,489 streptococci were included as part of the LEF Surveillance Program for 2015–2016. Isolates were tested for susceptibility by CLSI broth microdilution method. A total of 33 isolates with LEF MICs ≥0.5 µg/mL were selected for this study. Isolates had their bacterial genomes sequenced (MiSeq Sequencer, Illumina) and screened *in silico* for possible LEF resistance mechanisms.

Results: Many *Staphylococcus aureus* (7/11; 63.6%) harbored *vga*(A) (LEF MIC, 0.5–4 µg/mL), while 2 strains carried either *vga*(E) (LEF MIC, >32 µg/mL) or the enterococcal gene *lsa*(E) (LEF MIC, 32 µg/mL). One *S. aureus* had an alteration in L4 (E147K; LEF MIC, 16 µg/mL), whereas 1 isolate (LEF MIC, 0.5 µg/mL) did not have any of the known resistance mechanisms investigated. A total of 8 (57.1%) coagulase-negative staphylococci (CoNS) contained *vga* gene variants (LMU MIC, 1–8 µg/mL). Two *S. epidermidis* carried the *cfr* gene alone (LEF MIC, 4 µg/mL) or with multiple mutations in 23S rRNA, L3, and L4 (LEF MIC, 32 µg/mL). Two CoNS did not show any LEF resistance mechanisms (LEF MIC, 0.5 µg/mL), while 2 *S. sciuri* (LEF MIC, 16–32 µg/mL) carried the intrinsic putative *sal*(A) efflux pump gene. Many streptococci (10/13; 76.9%) carried *lsa*(E) (LEF MIC, 2–32 µg/mL), while 1 S. *anginosus* (LEF MIC, 1 µg/mL) had alterations in both L3 and L4. Two streptococci (LEF MIC, 0.5–0.25 µg/mL) had various alterations at 23S rRNA but outside the LEF binding site.

Conclusions: A small number of isolates (28 of 7,684; 0.36%) from global surveillance studies conducted in 2015–2016 had LEF MIC ≥0.5 µg/mL. The most common resistance mechanisms identified in staphylococci and streptococci were *vga* and *lsa*(E), respectively. Importantly, no *S. aureus* isolates harbored *cfr*. Global surveillance will be conducted to monitor changes over time.

INTRODUCTION

- Lefamulin is the first pleuromutilin antibiotic for intravenous (IV) and oral use in humans in late-stage clinical development to treat community-acquired bacterial pneumonia (CABP) and acute bacterial skin and skin structure infections (ABSSSI)
- This agent inhibits the bacterial protein synthesis by binding 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 as characteristic for pleuromutilin antibiotics
- Lefamulin recently demonstrated noninferiority to moxifloxacin ± linezolid in two phase 3 clinical trials for the treatment of CABP and was safe and well tolerated after IV and oral dosing
- The antibacterial profile of lefamulin covers the most relevant organisms causing CABP, including Gram-positive, fastidious Gram-negative, and atypical respiratory pathogens
- The *in vitro* activity of lefamulin and comparator agents has been monitored against a global collection of Gram-positive and fastidious Gram-negative organisms causing CABP and ABSSSI through the SENTRY Antimicrobial Surveillance Program
- This study evaluated the resistance mechanisms associated with elevated lefamulin minimum inhibitory concentration (MIC) values in a global collection of surveillance isolates from 2015 and 2016

MATERIALS AND METHODS

Bacterial Isolates

- A total of 3,195 staphylococci and 4,489 streptococci were included as part of the lefamulin surveillance program for 2015–2016
  - 25 Gram-positive isolates from 2015 and 2016 met the MIC screening criteria for molecular characterization with confirmed elevated lefamulin MIC results of ≥1 µg/mL (20 of 7,684; 0.26%; **Table 1**); an additional 5 *Staphylococcus* spp. and 3 viridans group streptococci with a lefamulin MIC value of 0.25–0.5 µg/mL were included
- 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 guidelines in the Clinical and Laboratory Standards Institute (CLSI) M07 (2018) document
- Frozen-form broth microdilution panels were manufactured by JMI Laboratories (North Liberty, IA) 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 (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Streptococcus 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 a MiSeq Sequencer (JMI Laboratories, North Liberty, IA, USA)
- FASTQ format sequencing files for each sample set were assembled independently using *de novo* assembler SPAdes 3.9.0, and an in-house designed software was applied to the assembled sequences to align against known macrolide, lincosamide, and streptogramin B (MLS_B) and pleuromutilin resistance genes
- Additional sequences of intrinsic genes associated with the pleuromutilin binding site, including 23S rRNA (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, and 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

- *S. aureus* selected for this study (lefamulin MIC, ≥0.5 µg/mL) comprised 11 isolates (0.3% and 0.4%) included in the respective 2015 and 2016 lefamulin surveillance programs, displaying a lefamulin MIC range of 0.5–>32 µg/mL **(Table 1)**
  - *S. aureus* isolates harboring the methyl transferase encoding gene *cfr* were not observed
  - 63.6% (7/11) of *S. aureus* isolates harbored *vga*(A) (lefamulin MIC, 0.5–4 µg/mL), while 2 strains carried either *vga*(E) (lefamulin MIC, >32 µg/mL) or *lsa*(E), which was also identified in *Streptococcus agalactiae* (lefamulin MIC, 32 µg/mL; **Tables 2 and 3**)
  - One *S. aureus* isolate had an alteration in L4 (E147K; lefamulin MIC, 16 µg/mL), whereas 1 isolate (lefamulin MIC, 0.5 µg/mL) did not have any of the known resistance mechanisms investigated **(Tables 2 and 3)**

Table 1. Lefamulin MIC Results Obtained During Surveillance Programs for 2015 and 2016

	No. of isolates with lefamulin MIC (µg/mL)																
Organism[a]	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32	Total	MIC_{50}	MIC_{90}
S. aureus	5	10	745	1,836	300	12	2	3	1	0	0	1		4	2,919	0.06	0.12
Coagulase-negative staphylococci	2	42	139	68	8	3	3	2	3	3	2	0		1	276	0.03	0.06
S. pneumoniae	5	64	377	1,715	1,513	234	10	5							3,923	0.06	0.12
S. agalactiae	1	21	136	4	3	0	0	0	0	0	2	1			168	0.03	0.03
S. anginosus	0	3	4	5	7	2	3								24		
S. anginosus group	1	1	1	3	3	0	1								10		
S. bovis	2	0	0	0	0	1									3		
S. constellatus	1	2	0	2											5		
S. dysgalactiae	0	3	45	8											56		
S. equinus	0	2													2		
S. gordonii					0	2									2		
S. intermedius	0	1	3	1											5		
S. lutetiensis	1	4	0	0	0	0	1	1							7		
S. mitis group	0	2	1	7	7	9	6								32		
S. mitis/oralis		1	0	1	2										4		
S. parasanguinis	0	2	2	2	1										7		
S. pyogenes	5	80	80												165		
S. salivarius	1	2	3	5	1										12		
S. salivarius group		0	1	8	2										11		
S. salivarius/vestibularis	4	2	2	6	2	1									17		
S. sanguinis				0	2	0	1								3		
S. gallolyticus			0	2	2	2	3	9	13	1	0	0		1	33	1	2

MIC=minimum inhibitory concentration; MIC_{50}=MIC at which 50% of the isolates were inhibited; MIC_{90}=MIC at which 90% of the isolates were inhibited.
[a]Clinical isolates meeting the MIC inclusion criteria and included in this study are highlighted.

Table 2. MIC Values Obtained for Lefamulin and Comparator Agents Tested Against Isolates Included in This Study

		MIC Values (µg/mL)								
Collection No.	Species	Lefamulin[a]	Clindamycin	Chloramphenicol	Linezolid	Q-D	Retapamulin	Oxacillin	Penicillin	Erythromycin
975498	*S. aureus*	0.5 (0.5)	>64	16	1	0.5	≤0.06	>2	>2	>8
981256	*S. aureus*	0.5 (0.5)	≤0.5	4	1	0.5	0.5	0.5	2	0.12
924825	*S. aureus*	1 (1)	≤0.5	8	1	0.5	2	>2	>2	>8
953474	*S. aureus*	1 (1)	≤0.5	4	0.5	0.5	1	≤0.25	≤0.06	>8
879822	*S. aureus*	2 (>1)	≤0.5	8	1	0.5	4	≤0.25	≤0.06	0.12
913640	*S. aureus*	2 (>1)	>64	8	1	0.5	2	>2	>2	>8
934242	*S. aureus*	2 (1)	≤0.5	8	0.5	0.5	2	≤0.25	0.12	0.12
950457	*S. aureus*	4 (2)	8	4	1	1	>8	>2	2	0.12
916083	*S. aureus*	16 (>1)	>64	8	0.25	1	8	>2	>2	>8
976441	*S. aureus*	32 (16)	>64	64	0.5	4	>8	0.5	>2	>8
972481	*S. aureus*	>32 (>16)	4	4	1	1	>8	>2	>2	4
939671	*S. cohnii*	0.5 (2)	≤0.5	4	1	1	1	1	0.25	>8
939504	*S. epidermidis*	0.5 (1)	≤0.5	16	16	≤0.25	0.25	>2	>2	>8
947675	*S. epidermidis*	1 (0.5)	16	4	0.5	≤0.25	>8	>2	>2	>8
951555	*S. epidermidis*	1 (0.5)	>64	4	0.5	4	1	>2	>2	>8
955639	*S. epidermidis*	1 (1)	≤0.5	4	0.5	0.5	1	>2	>2	0.12
956923	*S. epidermidis*	2 (0.5)	16	2	0.25	≤0.25	>8	>2	>2	>8
949426	*S. epidermidis*	2 (2)	1	4	0.5	≤0.25	>8	≤0.25	0.25	≤0.06
938399	*S. epidermidis*	8 (4)	≤0.5	2	0.5	≤0.25	8	>2	>2	>8
952506	*S. epidermidis*	8 (4)	1	4	0.5	≤0.25	8	>2	>2	>8
958510	*S. epidermidis*	8 (2)	2	4	1	≤0.25	>8	≤0.25	0.25	≤0.06
934123	*S. epidermidis*	32 (8)	>64	64	128	1	>8	>2	>2	0.5
939969	*S. haemolyticus*	4 (4)	>64	32	2	4	4	>2	>2	>8
944662	*S. sciuri*	16 (8)	>64	32	1	0.5	8	>2	>2	>8
941213	*S. sciuri*	32 (>16)	≤0.5	4	1	1	>8	>2	>2	0.25
973516	*S. bovis* group[b]	0.25 (0.25)	≤0.5	2	1	0.5	0.25	NT	0.06	>32
965031	*S. gallolyticus*[c]	32 (>16)	4	4	2	1	>8	NT	0.06	>32
960742	*S. lutetiensis*	0.5 (0.5)	≤0.5	2	1	1	0.5	NT	0.06	0.03
947639	*S. anginosus*	1 (0.5)	2	4	1	1	0.5	NT	0.03	≤0.015
982012	*S. lutetiensis*	2 (1)	1	2	1	0.5	2	NT	0.03	≤0.015
935554	*S. agalactiae*	16 (16)	4	2	0.5	0.5	4	NT	0.03	0.03
935557	*S. agalactiae*	8 (8)	4	2	1	0.5	4	NT	0.03	0.03
971459	*S. agalactiae*	8 (8)	>64	2	1	1	8	NT	0.06	>32

MIC=minimum inhibitory concentration; MIC_{50}=MIC at which 50% of the isolates were inhibited; MIC_{90}=MIC at which 90% of the isolates were inhibited; Q-D=quinupristin-dalfopristin; NT=not tested.
[a]Initial lefamulin MIC results obtained during the surveillance studies are within parentheses.
[b]*S. bovis* group was identified as *S. gallolyticus* subsp. *pasteurianus* based on genome sequence.
[c]*S. gallolyticus* was identified as *S. gallolyticus* subsp. *pasteurianus* based on genome sequence.

- A total of 14 coagulase-negative staphylococci (CoNS) with lefamulin MIC values of 0.5–>32 µg/mL were further characterized in this study
  - One *Staphylococcus cohnii* and 1 *Staphylococcus epidermidis* (lefamulin MIC, 0.5 µg/mL for both) did not show any lefamulin resistance mechanisms; only 23S rRNA alterations (C2534T and G2576T) known to affect the binding of oxazolidinones were observed **(Table 3)**
  - A total of 8 CoNS (57.1%) isolates contained the acquired *vga* gene variants (lefamulin MIC, 1–8 µg/mL)
  - One *Staphylococcus haemolyticus* isolate carried *cfr* alone (lefamulin MIC, 4 µg/mL), and 1 *S. epidermidis* isolate carried the *cfr* gene with multiple mutations in 23S rRNA, L3, and L4 (lefamulin MIC, 32 µg/mL)
  - Two *Staphylococcus sciuri* isolates (lefamulin MIC, 16–32 µg/mL) carried the intrinsic putative *sal*(A) efflux pump gene (data not shown)
  - Among *S. epidermidis* isolates, multiple alterations in the 23S rRNA were observed when compared with the wild-type control isolate **(Table 4)**; however, it is unclear whether these polymorphisms affected the susceptibility of pleuromutilin agents and all isolates had concomitant presence of *vga* or *cfr* **(Table 3)**
- Among the streptococci, elevated lefamulin MIC values were only observed within *S. agalactiae* (3 of 168) and 5 viridans group streptococci, whereas isolates with elevated MIC results were not observed among *S. pneumoniae*, *S. pyogenes*, and the majority of viridans group streptococcal species
  - All 3 *S. agalactiae* and the *S. lutetiensis* and *S. gallolyticus* subsp. *pasteurianus* isolates with lefamulin MIC values of 2–32 µg/mL **(Table 3)** carried *lsa*(E)
  - The *S. anginosus* isolate (lefamulin MIC of 1 µg/mL) had L3 and L4 alterations as well as multiple alterations in 23S rRNA outside the lefamulin binding site
  - The *S. bovis* group isolate 973516 displaying a lefamulin MIC value of 0.25 µg/mL was later identified as *S. gallolyticus* subsp. *pasteurianus* **(Tables 2 and 3)**. The lefamulin MIC of 0.25 µg/mL fits the distribution for *S. gallolyticus*
  - The *S. lutetiensis* isolate 960742 showed a 23S rRNA at 2359A **(Table 3)**

Table 3. Molecular Epidemiology and Resistance Mechanism Results for Isolates Included in This Study

						Resistance Determinants			Ribosomal Mutations[a]			
Species	Collection No.	Year	MLST[b]	Country	Lefamulin MIC, µg/mL	*cfr*	*lsa*(E)	*vga*[c]	23S rRNA	L3	L4	L22
S. aureus	975498	2016	5	USA	0.5	-	-	-	21G>A, 1557A>T	WT	WT	WT
S. aureus	981256	2016	4,335	New Zealand	0.5	-	-	*vga*(A)	21A>G, 1557A>T, 2234A>G	WT	WT	WT
S. aureus	924825	2015	88	Australia	1	-	-	*vga*(A)	21A>G, 1557A>T, 2234A>G	WT	WT	WT
S. aureus	953474	2016	398	France	1	-	-	*vga*(A)	21A>G, 1557A>T, 2234A>G	WT	WT	WT
S. aureus	879822	2015	1	Slovenia	2	-	-	*vga*(A)	21A>G, 2234A>G	WT	WT	WT
S. aureus	913640	2015	1,148	USA	2	-	-	*vga*(A)	21A>G, 2234A>G	WT	WT	WT
S. aureus	934242	2016	1,148	USA	2	-	-	*vga*(A)	21A>G, 2234A>G	WT	WT	WT
S. aureus	950457	2016	97	USA	4	-	-	*vga*(A)	21A>G, 2234A>G	WT	WT	WT
S. aureus	916083	2015	5	Korea	16	-	-	-	21A>G, 1557A>T, 2234A>G	WT	E147K	WT
S. aureus	976441	2016	398	Brazil	32	-	+	-	21A>G, 1557A>T, 2234A>G	WT	WT	WT
S. aureus	972481	2016	398	Germany	>32	-	-	*vga*(E)	21A>G, 1526A>G, 1557A>T	WT	WT	WT
S. cohnii	939671	2016	N/A[b]	USA	0.5	-	-	-	C2534T	WT	WT	WT
S. epidermidis	939504	2016	2	Italy	0.5	-	-	-	G2576T	WT	WT	WT
S. epidermidis	947675	2016	57	USA	1	-	-	*vga*(A)	[a]	WT	WT	WT
S. epidermidis	951555	2016	87	Czech Republic	1	-	-	*vga*(A), *vga*(B)	[a]	WT	WT	WT
S. epidermidis	955639	2016	87	Italy	1	-	-	*vga*(A), *vga*(B)	[a]	WT	WT	WT
S. epidermidis	956923	2016	679	Brazil	2	-	-	*vga*(A)	[a]	V188I	WT	WT
S. epidermidis	949426	2016	255	USA	2	-	-	*vga*(A)	[a]	WT	WT	WT
S. epidermidis	938399	2016	5	USA	8	-	-	*vga*(A)	[a]	WT	WT	WT
S. epidermidis	952506	2016	20	Argentina	8	-	-	*vga*(A)	[a]	WT	WT	WT
S. epidermidis	958510	2016	487	USA	8	-	-	*vga*(A)	[a]	WT	WT	WT
S. epidermidis	934123	2016	5	USA	32	+	-	-	C2534T	H146Q, V154L, A157R	G71_R72ins	WT
S. haemolyticus	939969	2016	3	Mexico	4	+	-	-	[a]	WT	WT	A29T
S. sciuri[d]	944662	2016	N/A[b]	Mexico	16	-	-	-	[a]	WT	WT	A112D
S. sciuri[d]	941213	2016	N/A[b]	Australia	32	-	-	-	[a]	WT	WT	WT
S. bovis group[e]	973516	2016	214	France	0.25	-	-	-	696C>T	WT	WT	WT
S. gallolyticus[f]	965031	2016	N/A	Spain	32	-	+	-	696C>T	WT	WT	WT
S. lutetiensis	960742	2016	N/A	Belgium	0.5	-	-	-	224T>C, 2359A>G	WT	WT	WT
S. anginosus	947639	2016	N/A[b]	USA	1	-	-	-	Multiple alterations[a]	A101E	I148L, S150T	WT
S. lutetiensis	982012	2016	N/A	Argentina	2	-	+	-	WT	WT	WT	WT
S. agalactiae	935554	2016	19	Mexico	16	-	+	-	WT	WT	WT	WT
S. agalactiae	935557	2016	19	Mexico	8	-	+	-	WT	WT	WT	WT
S. agalactiae	971459	2016	19	Korea	8	-	+	-	WT	WT	WT	WT

MLST=multilocus sequence typing; WT=wild type.
[a]23S rRNA mutational analysis was performed on nucleotide sequences. Additional mutations observed in *Staphylococcus* species other than *S. aureus* are described in **Table 4**. Alterations detected in *S. anginosus* were as follows: 150A>G, 152C>A, 164G>T, 434T>G, 957A>T, 1577T>C, 1866A>C, 1867C>G, 1872G>C, 1873T>G, and 2357A>T. Protein sequences were analyzed for annotating L3, L4, and L22.
[b]MLST method and database nonexistent.
[c]Includes *vga*(A), *vga*(A)LC, *vga*(B), *vga*(C), *vga*(D), and *vga*(E).
[d]The *sal*(A) gene was detected in both *S. sciuri* isolates.
[e]*S. bovis* group was identified as *S. gallolyticus* subsp. *pasteurianus*.
[f]*S. gallolyticus* was identified as *S. gallolyticus* subsp. *pasteurianus*.

Table 4. Polymorphisms Observed Within the 23S rRNA Among *Staphylococcus* spp. Other Than *S. aureus* Selected for This Study[a]

Organism	Year	Collection No.	23S rRNA
S. cohnii	2016	939671	333T>C, 1541T_1542GinsG, 1821A_1822GinsG, 2441C_2442GinsG, 2459T_2460CinsC, **2534T**_2535CinsC, 2762G_2763CinsC
S. epidermidis	2016	934123	669T>C,1236T>C, **2534T>C**
S. epidermidis	2016	938399	139C>T, 669T>C, 1236T>C
S. epidermidis	2016	939504	105G>A, 241G>T,669T>C, 1236T>C, **2576G>T**
S. epidermidis	2016	947675	241G>T, 669T>C, 1236T>C
S. epidermidis	2016	949426	669T>C, 1236T>C
S. epidermidis	2016	951555	139C>T, 241G>T, 669T>C, 1236T>C
S. epidermidis	2016	952506	139C>T, 669T>C, 1236T>C, 1638C>T
S. epidermidis	2016	955639	139C>T, 669T>C, 1236T>C, 2809C>T
S. epidermidis	2016	956923	669T>C, 1236T>C
S. epidermidis	2016	958510	241G>T, 669T>C, 1236T>C, 1638C>T
S. haemolyticus	2016	939969	1486C>T, 2235A>G, 2882T>C
S. sciuri	2016	941213	WT
S. sciuri	2016	944662	WT

WT=wild type.
[a]Bold selections represent alterations known to affect susceptibility of oxazolidinones.

CONCLUSIONS

- The overall resistance to lefamulin was very low and a small number of isolates (25 of 7,684; 0.33%) from global surveillance studies conducted in 2015–2016 had lefamulin MIC values ≥1 µg/mL
- Lefamulin resistance mechanisms identified in *S. aureus* isolates included *lsa*(E), *vga*(A), *vga*(E), and alteration in L4 (E147K); *vga* was the most common determinant observed
- No *S. aureus* isolates harbored *cfr*, whereas this was the case for 2 CoNS isolates from USA and Mexico
- Two *S. sciuri* isolates exhibiting elevated lefamulin MIC values (16–32 µg/mL) did not show any of the resistance mechanisms investigated
  - The intrinsic presence of *sal*(A) may be responsible for the elevated lefamulin MIC results in this species
- Although small in number, results from this study indicate that *vga* and *lsa* genes are the most common pleuromutilin resistance mechanisms in staphylococcal and streptococcal clinical isolates, respectively, and global surveillance will be conducted to monitor changes over time

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Disclosures

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