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Paradoxical High-Level Spiramycin Resistance and Erythromycin Susceptibility Due to 23S rRNA Mutation in *Streptococcus constellatus*

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1 **Paradoxical high-level spiramycin resistance and erythromycin**
2 **susceptibility due to 23S rRNA mutation in *Streptococcus***
3 ***constellatus***

4
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8
9 **Running title:** Paradoxical MLS resistance due to 23S rRNA mutation in
10 *Streptococcus constellatus*

11
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25

26 **Keywords:** *Streptococcus constellatus*; *Streptococcus milleri* group; MLS resistance;
27 23S rRNA mutation; A2062C; ARMS qPCR.

28 **Word counts:** Abstract: 253; Text: 1942; Tables: 2; Figures: 1; References: 19

29

30 **Abstract**

31 **Objectives:** The aim of the study was to characterize phenotypically and
32 genotypically an uncommon mechanism of resistance to macrolides, lincosamides
33 and streptogramins (MLS) in a *Streptococcus milleri* group clinical isolate.

34 **Methods:** The isolate UCN96 was recovered from an osteoradionecrosis wound, and
35 was identified using the MALDI-TOF mass spectrometry and the partial sequencing
36 of the *sodA* gene. Antimicrobial susceptibility testing (AST) were carried out by the
37 disc diffusion method and MICs were determined by the broth microdilution
38 technique. PCR screening was performed for MLS resistance genes described in
39 Gram-positive bacteria. Specific mutations in the ribosomal proteins L3-, L4- and
40 L22-encoding genes were also screened as well as those in domain V of the 23S
41 rRNA gene (*rrl*). The number of mutated copies of the *rrl* gene was determined using
42 ARMS (amplification-refractory mutation system) qPCR analysis.

43 **Results:** The clinical isolate UCN96 was unambiguously identified as *Streptococcus*
44 *constellatus*. It was susceptible to all ML antibiotics except spiramycin (MIC >256
45 mg/L) while it was also resistant to streptogramins. Screening for all acquired
46 resistance genes was negative while no mutation was found in genes coding for L3,
47 L4 and L22 ribosomal proteins. Interestingly, a single mutation, A2062C (according to
48 *Escherichia coli* numbering), was detected in the domain V of 23S rRNA.

49 **Conclusion:** Mutations at the position 2062 of 23S rRNA have been detected once
50 in *S. pneumoniae*, and not yet in other *Streptococcus* spp. This mechanism is very

51 likely uncommon in Gram-positive bacteria since different copies of 23S rRNA
52 operons should be mutated for development of such a resistance pattern.

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53 **Introduction**

54 *Streptococcus constellatus* belongs to the *Streptococcus milleri* group (SMG) besides
55 two others species, *Streptococcus anginosus* and *Streptococcus intermedius*. SMG
56 is part of the human microbiota and can be in the oral cavity as well as abdominal,
57 urogenital and upper respiratory tracts ¹. Streptococci from the SMG are responsible
58 for various pyogenic infections including cardiac, skin, abdominal and central nervous
59 system infections ¹. These organisms are usually susceptible to many antimicrobial
60 agents including those commonly used for infections caused by streptococci such as
61 β -lactams, macrolides-lincosamides-streptogramins (MLS) and glycopeptides.
62 Whereas there is no resistance to β -lactams and glycopeptides described in SMG,
63 acquired MLS resistance has been reported, with approximately 17% of clinical
64 isolates resistant to erythromycin and clindamycin ². In streptococci, MLS resistance
65 is mediated by two major mechanisms which are target site modification and active
66 drug efflux ³. Ribosomal alteration is caused by methylation of a specific adenine
67 residue (the so-called A2058) of target site in the 23S rRNA and is mediated by an
68 adenine-N6-methyltransferase encoded by a gene belonging to the *erm* class
69 (erythromycin ribosome methylase). This methylation of the A2058 residue induces a
70 conformational change in the 50S ribosomal subunit that alter the binding of
71 antibiotics to the ribosome and causes the so-called MLS_B phenotype. The second
72 mechanism consisted of an active efflux of the antibiotics encoded by the *mef* gene.
73 This active efflux is responsible for unique resistance to 14- and 15-membered
74 macrolides and confers the so-called M phenotype. Other mechanisms of MLS
75 resistance have been rarely described in Gram-positive bacteria, such as other
76 mutations in 23S rRNA or in L3, L4, L22 ribosomal proteins and to our knowledge,
77 have not been yet described in the SMG ⁴⁻⁸.

78 The aim of this study was to decipher the molecular aspect of an atypical
79 resistance phenotype in a clinical isolate of *S. constellatus* (UCN96).

80

81 (Part of this work was presented in the 14th European Congress of Clinical
82 Microbiology and Infectious Diseases, EV0187, Copenhagen, 2015)

83

84 **Materials & Methods**

85 ***Bacterial strains used in the study***

86 The UCN96 isolate was recovered in June 2014 from a osteoradionecrosis
87 radionecrosis wound in a patient hospitalized at the Gustave Roussy Institute
88 (Villejuif, France) for a tongue cancer medical history. The same bacterial strain was
89 recovered from the patient in a chin outpouring in May 2014 but the antimicrobial
90 susceptibility testing realized then, showed that the *S. constellatus* isolated was fully
91 susceptible to all MLS antibiotics. In front of this statement, the patient has received
92 pristinamycin at 1g twice in a day for 10 days since he was intolerant to β -lactams.
93 The isolate UCN96 was identified by MALDI-TOF mass spectrometry (Microflex;
94 Bruker Daltonics; Germany) and partial sequencing of the *sodA* gene as previously
95 described ⁹. *Staphylococcus aureus* ATCC 29213 and *Streptococcus pneumoniae*
96 ATCC 49619 were used as antimicrobial susceptibility testing (AST) controls while an
97 MLS-susceptible *S. constellatus* from our collection (*S. constellatus* 13422) was also
98 tested as wild-type control for comparative purposes.

99 ***Antimicrobial susceptibility testing***

100 Antimicrobial susceptibility testing was carried out by the disc diffusion method using
101 Mueller-Hinton agar plates supplemented with lysed horse blood (5%) and β -NAD
102 (20 mg/L) (bioMérieux; France) according to the Antibiogram Committee of the
103 French Society for Microbiology recommendations (CA-SFM/EUCAST; www.sfm-
104 microbiologie.org). MICs of erythromycin, azithromycin, spiramycin, josamycin,
105 telithromycin, lincomycin, clindamycin, pristinamycin, quinupristin-dalfopristin,
106 quinupristin, dalfopristin and chloramphenicol were determined by the broth
107 microdilution technique according to EUCAST guidelines (<http://eucast.org/>).

108

109 **DNA preparation, PCR assays and gene sequencing.**

110 DNA extraction was performed using the automated easyMAG® extractor
111 (bioMérieux, France) according to the protocol suggested by the manufacturer.
112 Extracted bacterial DNA was eluted with 25 µl elution buffer and stored at -20°C.
113 PCR experiments for the detection of acquired genes putatively involved in MLS
114 resistance [e.g. *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(TR), *erm*(T), *erm*(X), *msr*(A)
115 and *mef*(A)] as well as uncommon acquired genes [*Inu*(A), *Inu*(B), *Inu*(C), *Inu*(E),
116 *Isa*(A), *Isa*(B), *Isa*(C), *Isa*(E), *vat*(A), *vat*(B), *vat*(C), *vat*(D), *vat*(E), *vga*(A), *vga*(B),
117 *vga*(C), *vga*(D), *vga*(E), *vgb*(A), *vgb*(B)] were carried out using an in-house method.
118 The screening of point mutations was also performed by PCR-sequencing using
119 specific primers (Sigma-Aldrich, France) (Table 1) allowing amplification of the L3, L4
120 and L22 ribosomal protein encoding genes (*rplC*, *rplD* and *rplV* genes respectively)
121 and in the 23S rRNA (*rrl*) gene. Amplification refractory mutation system (ARMS)
122 qPCR was used to distinguished if the mutation of *rrl* gene affected all the alleles
123 harbored by the bacterial strain (e.g. 4 alleles in *S. constellatus* and all SMG) as
124 previously described in eucaryotes^{10,11}. Primers used for qPCR ARMS experiment
125 are detailed in Table 1. Briefly, two sets of primers were design. Oligonucleotide
126 sequences of the forward primers were strictly complementary to the *rrl* region where
127 the mutation was found. For one of the two forward primers, the nucleotide localized
128 at the 3' end corresponded to the nucleotide found in the mutant strain sequence,
129 whereas for the other, it corresponded to the nucleotide found in the wild type. The
130 reverse primer was the same for the 2 experiments. ARMS qPCR experiments were
131 made by real-time PCR using the SsoAdvanced universal SYBR green supermix kit
132 and a CFX Connect real-time PCR detection system (Bio-Rad, France) according to
133 manufacturer's recommendations. To determine if the mutation appeared in several

134 gene loci, a comparison of cycle threshold between the two sets of primers was
135 analyzed for the mutated strain UCN96. A susceptible strain of our collection, *S.*
136 *constellatus* 13422, was used as comparator. In case of higher initial amount of
137 mutated DNA matrix, a lower CT value should be obtained. Then if a difference in CT
138 values was observed between the 2 sets of primers, we should conclude that more
139 than one allele was mutated. Conversely, the same result was expected in favor of
140 the “wild-type” set of primers when using the *S. constellatus* 13244 DNA as template.

141

142 ***Multiple alignment, phylogenetic and gene analysis.***

143 Genome of the annotated strain *S. constellatus* 1050 available at the National Center
144 for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/genome/>) was used for
145 comparative sequences analysis. 23S rRNA gene partial sequence was then
146 compared to deposited sequences available from the BIBI site database
147 (<https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi>). Sequence comparison by
148 multiple alignment and phylogenetic analysis were performed using BioEdit
149 Sequence Alignment Editor Software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>)
150 and the neighbour-joining algorithm with the ClustalOmega software
151 (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

152

153 **Results**

154 ***Bacterial Identification***

155 The identification of strain UCN96 was carry out twice using the MALDI-TOF mass
156 spectrometry technology and was unambiguously confirmed as *S. constellatus* by the
157 partial *sodA* gene sequencing.

158

159 ***In vitro antimicrobial susceptibility testing***

160 By the disc diffusion method, the strain UCN96 was susceptible to almost all tested
161 antimicrobials: penicillin G, ampicillin, kanamycin, tobramycin, gentamicin,
162 vancomycin, teicoplanin, levofloxacin, rifampicin, tetracycline, chloramphenicol and
163 cotrimoxazole but in contrast, it exhibited an uncommon MLS resistance. After MIC
164 determination, the strain appeared harbouring an *a priori* paradoxical MS_{AB}
165 resistance phenotype. Indeed, the strain was susceptible to all 14- and 15-membered
166 macrolides (e.g. erythromycin, telithromycin, and azithromycin, with MICs of 0.03,
167 <0.008 and 0.12 mg/L, respectively), lincosamides (e.g. lincomycin and clindamycin,
168 with MICs of 1 and 0.06 mg/L, respectively) and chloramphenicol with a MIC of 8
169 mg/L. On the other hand, it was highly-resistant to spiramycin or josamycin (16-
170 membered macrolides) with MICs of >256 and 32 mg/L, respectively and
171 streptogramins (MIC >64 mg/L; 8 mg/L, 4 mg/L and 8 mg/L for dalfopristin,
172 quinupristin, quinupristin-dalfopristin and pristinamycin, respectively.) (Table 2). As
173 compared to *S. constellatus* 13422 susceptible strain, UCN96 exhibited MICs of
174 spiramycin, josamycin, dalfopristin, quinupristin, quinupristin-dalpristin and
175 pristinamycin 4, 512, 32, 8, 8 and 64-fold higher, respectively (Table 2).

176

177 ***Molecular characterization of the uncommon resistance phenotype***

178 PCR experiments for the detection of acquired genes putatively involved in MLS
179 resistance as well as uncommon acquired resistance patterns were all negative and
180 no mutation was found in L3, L4 and L22 ribosomal protein-encoding genes.
181 Interestingly, the strain displayed a point mutation in the *rrl* gene at an unexpected
182 position. Indeed, a nucleotide transition was found at position 2062 (*E. coli*
183 numbering) (A2062C) as shown in figure 1A. Considering that *S. constellatus*
184 harbored four copies of the ribosomal operon we assessed if the mutation affected
185 one or several *rrl* operons. The ARMS qPCR technique was used and confirmed the
186 presence of the A2062C mutation in more than a single copy of the 23S rRNA gene
187 in the strain UCN96. When DNA extracted from the resistant strain UCN96 was used
188 as template, cycle threshold (CT) obtained with the set of mismatches primers
189 appeared sooner (e.g. fourteen times), than the CT obtained with the set of primers
190 designed with the wild type sequence of the *rrl* operon (Figure 1B right). Inversely,
191 when the DNA extracted from the susceptible strain was used as template, only a six
192 CT difference was observed between the two sets of primers in favor of the
193 unmismatches primers (Figure 1B left). Such results, allow us to strongly suggest
194 that the point mutation A2062C was present in more than one 23S rRNA operon
195 copy.

196

197 **Discussion**

198 *S. constellatus* can be found as a part of the oral cavity and upper respiratory tract
199 flora but can be also responsible for various pyogenic infections where a therapeutic
200 management is required ¹. β -lactams are the first-line molecules recommended for
201 the treatment of streptococcal infections but in patients intolerant to that class of
202 antibiotics, MLS antimicrobials constitute an alternative therapy.

203 The target site for MLS molecules is the 50S ribosomal subunit ¹² and many cases of
204 MLS resistance could be linked with an alteration by methylation of 23S rRNA at
205 positions 2058 and/or 2059 ^{3,6}. In bacteria harboring several rRNA operon copies
206 (such as *Staphylococcus* spp., *Streptococcus* spp. or *Enterococcus* spp.) the most
207 prevalent mechanism for MLS resistance is an acquired mechanism consisting in
208 expression of *erm* gene encoding methyltransferase or active efflux of the antibiotics
209 encoded by the *mef* gene ^{3,7,13,14}. Point mutations in 23S rRNA have been reported
210 and appeared at specific positions of the domain V especially in pathogens harboring
211 only 1 or 2 rRNA operon copies, such as mycoplasma with a prevalence in
212 *Mycoplasma genitalium* estimated around 50% ¹⁵. These mutations, regarding the
213 species and nucleotide substitution, conferred different phenotypes of resistance. To
214 our best knowledge, MLS resistance linked to these types of mutations seemed to be
215 less prevalent in Gram-positive bacteria likely because they harbored a higher
216 number of rRNA operons (4 *rrn* operons in streptococci or enterococci for example)
217 and appeared generally at position 2058, 2059 or 2611, according to the species
218 involved ⁷. Interestingly, in our *S. constellatus* clinical isolate, the mutation found
219 (A2062C) seemed to be very uncommon since it has been reported only once in a
220 spiramycin- and streptogramin-resistant strain of *Streptococcus pneumoniae* and in a
221 chloramphenicol-resistant *Halobacterium halobium* ^{16,17,18}. Surprisingly, whereas *H.*

222 *halobium* harbored a single copy of rRNA operon, *S. pneumoniae* harbored 4 copies
223 (like *S. constellatus*) and the mutation was found in the four copies of this operon
224 ^{16,18,19}. Our data were in accordance with these observations since several copies of
225 *rfl* operon seemed altered in *S. constellatus* UCN96. Then, clinical isolates of
226 viridans streptococci that harbored this unusual phenotype of resistance
227 (susceptibility to 14-15 membered macrolides and lincosamides, resistance to 16-
228 membered macrolides and streptogramins) has not been reported yet. Moreover, as
229 previously described by different studies concerning the crystal structure of the
230 interaction area of MLS antibiotics with 50S SU, amino acids modification in position
231 2062 (transition to a nonpolar to a polar amino acid) possibility changed hydrogen
232 links between MLS antibiotics and their target in 50S SU of *Staphylococcus aureus*
233 ribosome. As a consequence, resistance to different MLS antibiotics should be
234 explained by this conformational variation ²⁰.

235 As a conclusion, we have identified and characterized a point mutation (A2062C)
236 found in more than one locus of the domain V of 23S rRNA that confers spiramycin
237 and streptogramins resistance in *S. constellatus* isolated from a patient suffering of
238 radionecrosis wound and treated with pristinamycin. Since *S. constellatus* is part of
239 the oral cavity and upper respiratory tract flora and that irradiation is largely use in
240 head and neck cancers, these data suggest that in clinical strains of SMG in patients
241 that previously received MLS therapy, it would be wise to follow the susceptibility of
242 the strain concerning all the MLS class of drugs (e.g. 14-, 15- and 16- membered
243 macrolide, ketolide and also to lincosamide and streptogramins).

244

245 **Author Disclosure Statement**

246 The authors declare that they have no conflicts of interest.

247

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251

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315

316

317 **Legend of the figure**

318

319 **Figure 1.** A) Partial nucleotide sequence alignment of 23S rRNA (*rrl*) encoding gene.
320 Mutation at position 2062 is framed in bold line (*E. coli* numbering). B Left) RT-PCR
321 results curves showing CT difference between wild-type (WT) primers vs ARMS

322 mismatches primers for a MLS susceptible strain of *S. constellatus* 13422 and the
323 resistant strain UCN96. Green lines corresponding to amplification when using WT
324 primers and red line corresponding to amplification when using mismatches primers.

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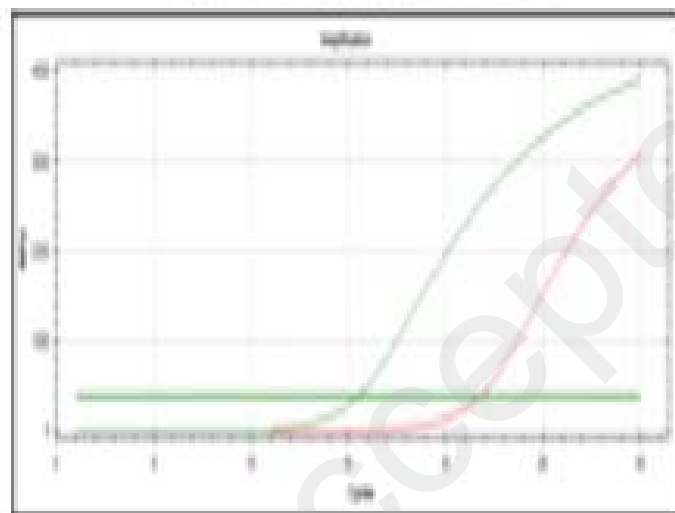
A)

```
23S_S. constellatus UCN96 CGGAAAGCCCCATGGAGCTTTACTGCAGTTTGATATTGAGTGTCTGTACCACATGTACA
23S_S. constellatus 1050 CGGAAAGCCCCATGGAGCTTTACTGCAGTTTGATATTGAGTGTCTGTGCCACATGTACA
23S_S. pneumoniae CGGAAAGCCCCATGGAGCTTTACTGCAGTTTGATATTGAGTGTCTGTACCACATGTACA
23S_E. coli CGGAAAGCCCCGTGAACCTTTACTATAGCTTGACACTGAACATTGAGCCTTGATGTGTA
```

2062

B)

S. constellatus 13422



S. constellatus UCN96

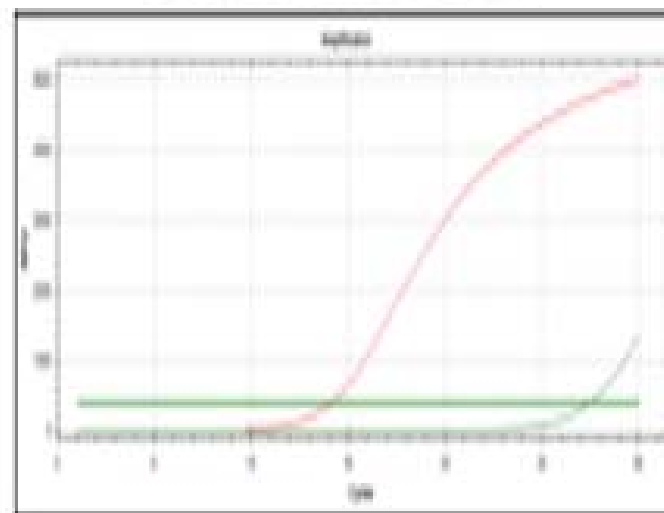


Table 1. Primers used in this study

Gene (ribosomal protein)	Primer		Purpose
	Designation	Sequence (5' to 3')	
<i>rplC</i> (L3)	L3-F	CGCCCTTGGTATCTTAATCT	Detection of point mutation in
	L3-R	CTTACCAGCTTCTTTACCAG	L3 protein
<i>rplD</i> (L4)	L4-F	GTGCCTGGTGCTAAGAAATC	Detection of point mutation in
	L4-R	TCTTCAAGAAGAGCCATTGA	L4 protein
<i>rplV</i> (L22)	L22-F	CACAAGCTTGGTGAGTTTGC	Detection of point mutation in
	L22-R	TACCATTTGGCATCCCAGTC	L22 protein
<i>rrl</i> (23S rRNA) domain V	23S-F	CGAAATTCCTTGTCGGGTAA	Detection of point mutation in
	23S-R	CCGTAGATGATCAACCTAC	domain V of 23S rRNA
<i>rrl</i> (23S rRNA) domain V	23S_ARMS_WT_F1	CGCGACAGGACGGAAAGA	Allelic determination of point mutation in 23S rRNA (ARMS)
	23S_ARMS_WT_F2	CGCGACAGGACGGAATGA	
	23S_ARMS_Mut_F1	CGCGACAGGACGGAAAGC	
	23S_ARMS_Mut_F2	CGCGACAGGACGGAATGC	
	23S_ARMS_R	ATCCCAACATCGCCTCCATC	

Table 2. MICs of MLS antibiotics and Chloramphenicol used in this study

Strains	MIC ($\mu\text{g/ml}$)											
	Macrolides					Lincosamides		Streptogramins			Phenicol	
	ERY	TEL	AZI	SPI	JOS	LIN	CLI	DAL	QUI	QD	PRI	CHL
<i>S. constellatus</i> 13422	<0.01	<0.01	0.06	0.06	0.06	0.5	0.03	2	1	0.5	0.12	2
<i>S. constellatus</i> UCN96	0.03	<0.01	0.12	≥ 256	32	1	0.06	≥ 64	8	4	8	8
<i>S. aureus</i> 29213	0.25	0.06	1	8	2	1	0.25	4	2	0.5	0.25	8
<i>S.</i> <i>pneumoniae</i> 49619	0.06	<0.01	0.12	0.25	0.06	0.5	0.06	16	1	0.25	0.12	2

ERY, Erythromycin ; TEL, Telithromycin ; AZI, Azithromycin ; SPI, Spiramycin ; JOS, Josamycin ; LIN, Lincomycin ; CLI, Clindamycin ;

DAL, Dalfopristin ; QUI, Quinupristin ; Q-D, Quinupristin-Dalfopristin ; PRI, Pristinamycin ; CHL, Chloramphenicol.