Addition of Daptomycin to Levofloxacin Increased the Efficacy of Levofloxacin Monotherapy against a Methicillin-Susceptible *Staphylococcus aureus* Strain in Experimental Meningitis and Prevented Development of Resistance

Authors

Philippe Cottagnoud^{1*}, Frederike Sprenker¹, Marianne Cottagnoud², Alexandra Collaud³,
Reza Ashkbus¹, Vincent Perreten³

¹, Ärztezentrum Südbahnhof, Bern, Switzerland

², former Spitalnetz Bern, Bern, Switzerland

³, Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern,
Switzerland

*corresponding author:

Address: Ärztezentrum Südbahnhof, Chutzenstrasse 24, CH-3007 Bern

E-mail: philippe.cottagnoud@hin.ch

19 **Abstract**

20 Daptomycin and levofloxacin were tested as monotherapies and in combination against the
 21 antibiotic-susceptible *S. aureus* strain MSSA 1112 in a rabbit meningitis model and the effect
 22 of the combination on induction of resistance was determined in vitro. Changes of the
 23 susceptibility to fluoroquinolones and daptomycin was determined by the measurement of
 24 the MIC and mutations were detected by whole genome sequence comparison of the
 25 mutants with the parent strain MSSA 1112. Meningitis was induced by intracisternal
 26 inoculation of 10^5 CFU of MSSA 1112 and treatment was started 10 h later by injection of
 27 daptomycin (15 mg/kg) and levofloxacin (10 mg/kg) standard doses. Cerebrospinal fluid
 28 (CSF) samples were repeatedly collected during therapy in order to determine killing rates
 29 and results of bactericidal activity were expressed in $\Delta\log_{10}$ CFU/ml over 8 h. The
 30 combination of daptomycin with levofloxacin was significantly ($p < 0.001$) superior to
 31 levofloxacin monotherapy and increased the antibacterial activity of daptomycin. In vitro,
 32 MSSA 1112 was cycled over six days with either increasing concentrations of levofloxacin or
 33 daptomycin or with a combination of levofloxacin with half of the MIC of daptomycin or
 34 daptomycin with half of the MIC of levofloxacin leading to mutations in target genes as
 35 identified by whole genome sequence analysis. Addition of low concentration of daptomycin
 36 (0.25 mg/L) reduced levofloxacin-induced resistance in vitro. Addition of levofloxacin in low
 37 concentration (0.125 mg/L) did not influence daptomycin-induced resistance.
 38 These findings highlight the lack of reciprocal interference of antibiotics in combination with
 39 regard to the development of resistance.

40

41 Introduction

42 The treatment of meningitis caused by *Staphylococcus aureus*, occurring mainly after
43 surgical interventions, remains a major challenge for clinicians and infectiologists (1). By
44 now, the treatment of choice against methicillin-susceptible *S. aureus* (MSSA) strains is
45 based on β -lactam antibiotics, and in case of penicillin allergy, vancomycin is usually used
46 (2). Due to the adverse effects of vancomycin, i.e. nephrotoxicity and Red Neck syndrome,
47 an alternative regimen with bactericidal activity is needed and would represent a
48 considerable progress. Levofloxacin has been shown to have a good activity in
49 pneumococcal meningitis (3) and daptomycin was efficacious as monotherapy in
50 experimental meningitis caused by MSSA (4). However, development of resistance in *S.*
51 *aureus* during monotherapies is of major concern(5, 6) . Resistance of *S. aureus* to
52 fluoroquinolones mainly occurs through mutations in the quinolone resistance-determining
53 regions (QRDR) of the topoisomerase II (GyrA and GyrB) and IV (GrlA and GrlB) (7, 8). The
54 mechanism of daptomycin resistance is still not well understood, but mutations and up-
55 regulation of mostly cell wall related genes such as those encoding for phosphatidylglycerol
56 lysyltransferase MprF, the two-component cell wall stress response associated regulator
57 WalKR, the cardiolipin synthetase CIs2, the peptidoglycan synthesis associated proteins
58 PgsA and MurA1/2 have been shown to be associated with daptomycin resistance in *S.*
59 *aureus* (8-13). Additionally, mutations in the staphyloxanthin synthesis gene *crtM*, as well as
60 deletion of the *rpsU* gene encoding for the 30S ribosomal protein S21, of the hypothetical
61 lipoprotein Dsp1, and of the alkaline shock protein Asp23 also lead to decreased
62 susceptibility to daptomycin (11, 14).

63 Addition of β -lactam antibiotics in subminimal inhibitory concentration (sub-MIC) has been
64 shown to prevent the emergence of quinolone resistance in an experimental rabbit meningitis
65 model using pneumococcal strains (3, 15). Such previous observations prompted us to
66 analyze the efficacy of levofloxacin and daptomycin monotherapies and of a combination
67 thereof against a methicillin-susceptible *S. aureus* strain using the same experimental
68 meningitis model. Additionally, we evaluated the effect of the combination of levofloxacin and

daptomycin in cycling experiments in vitro on the emergence of resistance against both antibiotics and searched for mutations by comparative analysis of whole genome sequence of the mutants with the parent strain.

72

73 **Material and Methods**

74 **Rabbit Meningitis Model**

75 Induction of meningitis with methicillin-susceptible *S. aureus* strain MSSA 1112 and CSF
76 sampling were performed in Young New Zealand White rabbits as described previously (3,
77 16). Strain MSSA 1112 was initially isolated from the blood sample of a patient with
78 endocarditis and was susceptible to levofloxacin (MIC 0.25 mg/L) and daptomycin (MIC 0.5
79 mg/L) (17). Animals were anesthetized by intramuscular injections for induction of meningitis
80 with 10⁵ CFU of MSSA 1112 instilled in the cisterna magna and for CFU sampling from CSF.
81 The experimental protocol was approved by the ethical committee of the Canton of Bern,
82 Switzerland (Veterinäramt des Kantons Bern). The cisterna magna was punctured again for
83 periodic CSF sampling before and 1, 2, 4, 5, 6, and 8 hours after initiation of therapy.
84 Daptomycin (15 mg/kg) and levofloxacin (10 mg/kg) were administered by a peripheral ear
85 vein injection once 14h after induction of meningitis.
86 Colony forming unit (CFU) of MSSA 1112 was determined by 10-fold serial dilutions of CSF
87 samples plated on Muller-Hinton agar plates and incubated overnight at 37° C. Twenty
88 microliters of undiluted samples were also plated to allow a limit of detectability of 50
89 CFU/ml). The dilutions of CSF were compared to exclude significant carryover effects during
90 therapy. The antimicrobial activity of the antibiotics during the 8-h treatment was calculated
91 by linear regression analysis and expressed as change in log₁₀ CFU per milliliter per hour
92 and as change of viable count over 8 h. A value of 1.7 (log₁₀ of the limit of detectability) was
93 assigned to the first sterile CSF sample, and a value of 0 was assigned to any following
94 sterile CSF sample. The results were interpreted as means ± standard deviation and
95 statistical significance was determined using the Newman-Keuls method (18).

96 **Selection of levofloxacin and daptomycin-resistant derivatives in vitro**

97 The tendency of levofloxacin and daptomycin, alone or in combination, to select for resistant
98 mutants of MSSA 1112 was tested in Mueller-Hinton broth. Inocula of 10^7 - 10^8 CFU/ml MSSA
99 1112 were exposed to serial two-fold increasing concentrations of levofloxacin or
100 daptomycin. After 12 hours of incubation at 37°C, 0.1 ml of the cultures from the tubes
101 containing the highest antibiotic concentration and still showing turbidity were used to
102 inoculate a new series of tubes containing antibiotic serial dilutions. The experiments were
103 performed over 12 cycles, and the minimal inhibitory concentration (MIC) was determined at
104 the end of the experimental period.

105 The same method was used either with daptomycin added in low concentration (0.25 mg/L
106 corresponding to half the MIC (1/2 MIC) to the tubes containing two-fold serial dilutions of
107 levofloxacin or with levofloxacin (1/2 MIC, 0.125 mg/L) to the tubes containing two-fold serial
108 dilutions of daptomycin. The experiments were performed during 12 cycles.

109 **Antimicrobial susceptibility testing of the parent and mutant strains**

110 Antimicrobial susceptibility was determined for *S. aureus* MSSA 1112 and derived mutants
111 (MSSA 1112-2, MSSA 1112-3, MSSA 1112-4 and MSSA 1112-5) by the measurement of the
112 MIC of ampicillin, ciprofloxacin, daptomycin, erythromycin, tetracycline, gentamicin, linezolid,
113 quinopristin-dalfopristin, teicoplanin, tigecycline, and vancomycin in Mueller-Hinton broth
114 using sensititre plates EUVENC Sensititre® plates (Thermo Fisher Scientific, Waltham, USA)
115 and home-made 96-well microtiter plates for levofloxacin following the CLSI guidelines (19).

116 **Whole genome sequencing and detection of mutations associated with increased** 117 **resistance to antibiotics**

118 Whole genome sequencing (WGS) of *S. aureus* strain MSSA 1112 was performed with both
119 long and short reads to obtain a complete and accurate genome to be used as scaffold.
120 WGS of the 4 MSSA 1112 mutants (MSSA 1112-2 to MSSA 1112-5) was made with short
121 reads which were used for read mapping against the scaffold of MSSA 1112. Short reads
122 were obtained using NovaSeq6000 S2 Reagent Kit (300cycles) and S2 flow cell on a
123 NovaSeq6000 (2x150-bp paired-end) system (Illumina, USA) at Eurofins Genomics GmbH,

Germany. The adaptors and low-quality reads ($QV \leq 20$) of the short reads were removed using Trimmomatic v0.36 (20). Long read sequencing of MSSA 1112 was performed using Oxford Nanopore Technologies (ONT) (ONT, United Kingdom). The library was prepared using the Ligation Sequencing Kit 1D SQK-LSK108 in an R9.4 SpotON flow cell with a MinION Mk1B device (ONT). The reads were base-called using Guppy basecaller (v2.3.7) (ONT). Hybrid *de novo* assembly of both short and long reads of MSSA 1112 was obtained using Unicycler v0.4.4 (21), and the resulting assembled genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (22). Detection of mutations and read mapping of the Illumina reads of the 4 mutants (MSSA 1112-2 to MSSA 1112-5) against the genome of the parent strain MSSA 1112 was performed using Geneious Prime® 2019.0.4 (Biomatters Ltd, Auckland, New Zealand). DNA sequence of genes containing mutations was translated into amino acid sequences to identify non-silent mutations at the protein level.

RESULTS

The efficacies of the different antimicrobial regimens in experimental rabbit meningitis are presented in Table 1. At the beginning of the experimental period, the inocula were similar in all treatment groups. As described previously, untreated controls grew slowly and showed only a slight increase of the CFU after 8 hours ($+ 0.60 \pm 0.43 \log_{10}$ CFU/ml) (4). Daptomycin monotherapy was bactericidal and showed a decrease of $- 0.48 \pm 0.13 \log_{10}$ CFU/ml per hour and a decrease of $- 4.06 \pm 1.23 \log_{10}$ CFU/ml after 8 hours. Levofloxacin monotherapy was slightly less efficacious with killing rates of $0.38 \pm 0.05 \log_{10}$ CFU/ml per hour and $3.47 \pm 0.55 \log_{10}$ CFU/ml at the end of the experimental period. Combination of both antibiotics was the most potent regimen with killing rates of $0.87 \pm 0.09 \log_{10}$ CFU/ml per hour and $4.81 \pm 0.69 \log_{10}$ CFU/ml after 8 hours, respectively. At the end of the experimental period, levofloxacin alone was not sufficient to clear the CSF of the rabbits from bacterial cells. Two of 5 CSF were sterile in the daptomycin group and 4 of 5 CSF in the combination group, respectively.

152

153 Six days of cycling with levofloxacin alone led to an eightfold increase of the MIC (2 mg/L).
 154 The addition of daptomycin in subinhibitory concentration (0.25 mg/L corresponding to 1/2
 155 MIC) to levofloxacin cultures drastically reduced the increase of the MIC of levofloxacin (0.5
 156 mg/L). Also after six days of cycling with increasing concentrations of daptomycin alone, the
 157 MIC of daptomycin increased to 4 mg/L. In contrast, the addition of low dose of levofloxacin
 158 (0.125 mg/L corresponding to 1/2 MIC) to daptomycin cultures did not influence the increase
 159 of the MIC of daptomycin (8 mg/L) (Table 2). In addition, the MIC for vancomycin was
 160 determined for all groups. Interestingly, cycling MSSA 1112 cultures with daptomycin led to a
 161 slightly increase of the MIC of vancomycin (MIC 2 mg/L). Even the addition of daptomycin in
 162 low dose to levofloxacin induced the same increase of MIC of vancomycin. On the other
 163 hand, levofloxacin monotherapy did not influence the MIC of vancomycin (Table 2). No
 164 change in the MIC of the other antibiotics tested was observed.

165 The genome of the parent strain MSSA 1112 and the mutants was sequenced to identify
 166 mutations resulting in the mutants after the different antibiotic cycles (Table 2).

167 Mutations associated with fluoroquinolone resistance were found in the QRDR of the
 168 topoisomerases IV (GrlA) and II (GyrB) of MSSA 1112-3 after cycling of MSSA 1112 with
 169 levofloxacin monotherapy. In mutant MSSA 1112-5 generated in the cycling group containing
 170 increasing concentrations of levofloxacin combined with a low dose of daptomycin, mutations
 171 resulted in amino acid substitutions in the QRDR of GrlB with slight increase of the
 172 levofloxacin MIC to 0.5 mg/L, and to amino acid substitutions in FabI, as well as frameshift in
 173 RplU and TlyC resulting in an early stop codon and an extension of the protein sequence,
 174 respectively. None of these mutations affected the MIC of daptomycin which remained the
 175 same as the parent strain (Table 2). The two mutants MSSA 1112-2 and MSSA 1112-4
 176 exhibiting decreased susceptibility to daptomycin harbored several different mutations.

177 Mutant MSSA 1112-2 which was selected on daptomycin only harbored amino acid
 178 substitutions in MrpF, Cls2 and Spa, as well as frameshift-resulting mutations in AroK.

179 Mutant MSSA 1112-4 which was selected with increasing concentration of daptomycin

combined with low dose levofloxacin, exhibited an amino acid substitution in GrlA and two frameshift-resulting mutations in the *ypdA* and *rpsU* genes, both leading to an early stop codon in the respective proteins YpdA and RpsU (Table 2).

Discussion

In previous studies, we have shown that β -lactam antibiotics, i.e. ceftriaxone, cefotaxime, and meropenem were very active especially in combination with levofloxacin in experimental pneumococcal meningitis (3, 15, 23). These studies also showed that addition of β -lactam antibiotics reduced the emergence of levofloxacin-induced resistance in pneumococci demonstrated in cycling experiments in vitro. In the present study, a similar experimental setting was used to test the efficacy of daptomycin combined with levofloxacin compared to the monotherapies against a MSSA strain in experimental meningitis. Furthermore, the potency of the combination to modify the emergence of resistance compared to the monotherapies was evaluated in vitro. As expected from previous experiments (4), bacterial growth was slow in the untreated controls over 8 hours. Daptomycin was slightly more efficacious than levofloxacin although the difference was statistically not significant. Only the combination treatment was clearly superior to the monotherapies, especially to levofloxacin ($P < 0.001$). Probably a higher number of animals in each group would have been more discriminating, especially between the two monotherapies. Nevertheless, daptomycin as monotherapy or in combination with quinolones, seems to be a promising therapy of meningitis caused by MSSA.

In cycling experiments in vitro, incubation of the MSSA 1112 strain either with levofloxacin or daptomycin led to a pronounced increase of the MIC for the two monotherapies. The MIC increases were confirmed by detection of mutations in the target genes. Levofloxacin induced resistance was confirmed by the presence of a S80F substitution in GrlA, which is very common among fluoroquinolone-resistant clinical *S. aureus* isolates (7). An additional amino acid substitution was detected in GyrB (W592F) and its role in increased MIC of

levofloxacin is not known. The mutant selected under daptomycin contained four different mutations, including amino acid substitutions in MprF (P314L) and Cls2 (A23V), which correspond to known mutations associated with resistance to daptomycin resistance in clinical *S. aureus* isolates and in in vitro generated mutants (9, 10, 24). The two other mutations were detected in the shikimate kinase AroK and the immunoglobulin G-binding protein A Spa, whose associations with decreased susceptibility to daptomycin are not known. The most striking feature of the study was the effect of the combination treatments on the emergence of resistance to the monotherapies. Addition of low-dose daptomycin to cultures cycled with levofloxacin reduced the development of resistance to levofloxacin leading only to a twofold increase of MIC of levofloxacin. In this mutant (MSSA 1112-5) only one amino acid substitution was detected in GrIB (L440V) which is situated within the QRDR of GrIB and may explain this slight increase of MIC (25). This mutant obtained in the presence of low dose of daptomycin harbored additional mutations, i.e. frameshift mutations in RplU, FabI, PflA and TlyC genes without influencing the MIC for daptomycin. Obviously these mutations are of minor relevance in creating daptomycin resistance. On the other hand, addition of low dose levofloxacin to cultures cycled with daptomycin did not influence the daptomycin-induced resistance. In this mutant, frameshift mutations were detected in pyridine nucleotide-disulfide oxidoreductase YpdA and the 30S ribosomal protein S21 (RpsU). While deletion of RpsU is known to confer resistance to daptomycin in *S. aureus* (14), the association of a frameshift mutation in YpdA with daptomycin resistance is so far not known. In this mutant, a mutation in GrIA was also induced by low dose levofloxacin, correlating with the slight increase of its MIC.

Another interesting feature was the effect of daptomycin selection on the slight increase of the MIC of vancomycin. The underlying mechanism of this resistance development affecting these two antibiotics with a different bacterial target (cell wall synthesis versus integrity of cell membrane) is not well understood. However, simultaneous decreased susceptibility of *S. aureus* to vancomycin and daptomycin during either vancomycin or daptomycin treatment has been reported in clinical isolates (26-29) (REF) (REF). Specific mutations in MprF have

been shown to affect non-susceptibility to both daptomycin and vancomycin (30, 31). However, the MprF mutation (P314L) generated in the MSSA 1112-2 mutant has so far only been shown to be associated with non-susceptibility to daptomycin, both not to vancomycin (9). Additionally, the role of the remaining mutations in Cls2, AroK, Spa, YpdA, RpsU, RplU, PflA, FabI, TlyC generated under daptomycin selection in vancomycin non-susceptibility still remains to be determined. In conclusion, daptomycin combined with levofloxacin seemed to be an efficacious and promising treatment for bacterial meningitis due to MSSA. In vitro, daptomycin prevented or decreased levofloxacin-induced resistance. On the other side, levofloxacin did not manage to influence daptomycin-induced resistance. Of note, both antibiotics added in low dose in the combination groups were able to induce mutations in their target genes emphasizing the importance of prudent and appropriate use of antibiotics.

ACKNOWLEDGEMENTS

We thank Dr. J. Entenza and Dr. P. Moreillon of the Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland, who kindly provided *S. aureus* strain MSSA 1112.

References

1. Aguilar J, Urday-Cornejo V, Donabedian S, Perri M, Tibbetts R, Zervos M. 2010. Staphylococcus aureus meningitis: case series and literature review. *Medicine (Baltimore)* 89:117-25.
2. John J, Jr. 2020. The treatment of resistant staphylococcal infections. *F1000Res* 9.
3. Flatz L, Cottagnoud M, Kuhn F, Entenza J, Stucki A, Cottagnoud P. 2004. Ceftriaxone acts synergistically with levofloxacin in experimental meningitis and reduces levofloxacin-induced resistance in penicillin-resistant pneumococci. *J Antimicrob Chemother* 53:305-10.
4. Gerber P, Stucki A, Acosta F, Cottagnoud M, Cottagnoud P. 2006. Daptomycin is more efficacious than vancomycin against a methicillin-susceptible Staphylococcus aureus in experimental meningitis. *J Antimicrob Chemother* 57:720-3.
5. Sabat AJ, Tinelli M, Grundmann H, Akkerboom V, Monaco M, Del Grosso M, Errico G, Pantosti A, Friedrich AW. 2018. Daptomycin Resistant Staphylococcus aureus Clinical Strain With Novel Non-synonymous Mutations in the mprF and vraS Genes: A New Insight Into Daptomycin Resistance. *Front Microbiol* 9:2705.
6. Kashyap R SA, Dutt T, Wieruszewski PM, Ahdal J, Jain R. . 2019. Treatments and limitations for methicillin-resistant Staphylococcus aureus: A review of current literature. *World J Clin Infect Dis* 9(1): 1-1
7. Fuzi M, Rodriguez Bano J, Toth A. 2020. Global Evolution of Pathogenic Bacteria With Extensive Use of Fluoroquinolone Agents. *Front Microbiol* 11:271.
8. Hooper DC, Jacoby GA. 2016. Topoisomerase Inhibitors: Fluoroquinolone Mechanisms of Action and Resistance. *Cold Spring Harb Perspect Med* 6.
9. Ernst CM, Peschel A. 2019. MprF-mediated daptomycin resistance. *Int J Med Microbiol* 309:359-363.
10. Gomez Casanova N, Siller Ruiz M, Munoz Bellido JL. 2017. Mechanisms of resistance to daptomycin in Staphylococcus aureus. *Rev Esp Quimioter* 30:391-396.
11. Barros EM, Martin MJ, Selleck EM, Lebreton F, Sampaio JLM, Gilmore MS. 2019. Daptomycin Resistance and Tolerance Due to Loss of Function in Staphylococcus aureus dsp1 and asp23. *Antimicrob Agents Chemother* 63.
12. Miller WR ea. 2016. Mechanism of Action and Resistance to Daptomycin in Staphylococcus aureus and Enterococci. *Cold Spring Harb Perspect Med*.
13. Coe KA LW, Stone MC, Komazin-Meredith G, Meredith TC, Grad YH, Walker S. P. 2019. Multi-strain Tn-Seq reveals common daptomycin resistance determinants in Staphylococcus aureus. *PLoS Pathog* Nov 18;15(11).
14. Blake KL, O'Neill AJ. 2013. Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents. *J Antimicrob Chemother* 68:12-6.
15. Kuhn F, Cottagnoud M, Acosta F, Flatz L, Entenza J, Cottagnoud P. 2003. Cefotaxime acts synergistically with levofloxacin in experimental meningitis due to penicillin-resistant pneumococci and prevents selection of levofloxacin-resistant mutants in vitro. *Antimicrob Agents Chemother* 47:2487-91.
16. Dacey RG, Sande MA. 1974. Effect of probenecid on cerebrospinal fluid concentrations of penicillin and cephalosporin derivatives. *Antimicrob Agents Chemother* 6:437-41.
17. Entenza JM, Vouillamoz J, Glauser MP, Moreillon P. 1997. Levofloxacin versus ciprofloxacin, flucloxacillin, or vancomycin for treatment of experimental endocarditis due to methicillin-susceptible or -resistant Staphylococcus aureus. *Antimicrob Agents Chemother* 41:1662-7.
18. Wu SS, Wang W, Annis DH. 2008. On identification of the number of best treatments using the Newman-Keuls test. *Biom J* 50:861-9.

19. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 11th ed. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute; 2018).
20. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114-20.
21. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 13:e1005595.
22. Tatusova T, DiCuccio M, Badretin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M, Ostell J. 2016. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* 44:6614-24.
23. Cottagnoud P, Cottagnoud M, Acosta F, Flatz L, Kuhn F, Stucki A, Entenza J. 2003. Meropenem prevents levofloxacin-induced resistance in penicillin-resistant pneumococci and acts synergistically with levofloxacin in experimental meningitis. *Eur J Clin Microbiol Infect Dis* 22:656-62.
24. Erica Lasek-Nesselquist JL, Ryan Schneider , Zhuo Ma , Vincenzo Russo , Smruti Mishra , Manjunath P Pai , Janice D Pata , Kathleen A McDonough , Meenakshi Malik 2019. Insights Into the Evolution of *Staphylococcus aureus* Daptomycin Resistance From an in vitro Bioreactor Model *Front Microbiol* Feb 28;10:345.
25. Schmitz FJ, Kohrer K, Scheuring S, Verhoef J, Fluit A, Heinz HP, Jones ME. 1999. The stability of *grlA*, *grlB*, *gyrA*, *gyrB* and *norA* mutations and MIC values of five fluoroquinolones in three different clonal populations of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 5:287-290.
26. Moise PA, North D, Steenbergen JN, Sakoulas G. 2009. Susceptibility relationship between vancomycin and daptomycin in *Staphylococcus aureus*: facts and assumptions. *Lancet Infect Dis* 9:617-24.
27. Patel JB, Jevitt LA, Hageman J, McDonald LC, Tenover FC. 2006. An association between reduced susceptibility to daptomycin and reduced susceptibility to vancomycin in *Staphylococcus aureus*. *Clin Infect Dis* 42:1652-3.
28. Lee CH, Wang MC, Huang IW, Chen FJ, Lauderdale TL. 2010. Development of daptomycin nonsusceptibility with heterogeneous vancomycin-intermediate resistance and oxacillin susceptibility in methicillin-resistant *Staphylococcus aureus* during high-dose daptomycin treatment. *Antimicrob Agents Chemother* 54:4038-40.
29. Kelley PG, Gao W, Ward PB, Howden BP. 2011. Daptomycin non-susceptibility in vancomycin-intermediate *Staphylococcus aureus* (VISA) and heterogeneous-VISA (hVISA): implications for therapy after vancomycin treatment failure. *J Antimicrob Chemother* 66:1057-60.
30. Feng-Jui Chen T-LL, Chen-Hsiang Lee , Yu-Chieh Hsu , I-Wen Huang , Pei-Chi Hsu , Chung-Shi Yang 2018. Effect of a Point Mutation in *mprF* on Susceptibility to Daptomycin, Vancomycin, and Oxacillin in an MRSA Clinical Strain *Front Microbiol* 25;9:1086.
31. Thitiananpakorn K, Aiba Y, Tan XE, Watanabe S, Kiga K, Sato'o Y, Boonsiri T, Li FY, Sasahara T, Taki Y, Azam AH, Zhang Y, Cui L. 2020. Association of *mprF* mutations with cross-resistance to daptomycin and vancomycin in methicillin-resistant *Staphylococcus aureus* (MRSA). *Sci Rep* 10:16107.

Table 1. Efficacy of the different antibiotic regimens against methicillin-susceptible *S. aureus* in experimental meningitis

Groups (N) [Strain]	Inoculum (log ₁₀ CFU/ml)	Killing rates/h (Δ log ₁₀ CFU/ml·h)	Killing rates/8h (Δ log ₁₀ CFU/ml·8h)
Controls (5) [MSSA]	5.53 ± 0.39	+ 0.08 ± 0.06	+ 0.60 ± 0.43
DAP (5) [MSSA]	5.41 ± 0.37	- 0.48 ± 0.13 ^{1,2}	- 4.06 ± 1.23 ⁵
LVX (5) [MSSA]	5.54 ± 0.33	- 0.38 ± 0.05 ^{1,3}	- 3.47 ± 0.55 ⁴
LVX+DAP (5) [MSSA]	5.21 ± 0.46	- 0.87 ± 0.09 ^{2,3}	- 4.81 ± 0.69 ^{4,5}

1: LVX versus DAP: p<0.17, not significant

2: LVX+DAP versus D: p=0.08, not significant trend

3: LVX+DAP versus LVX: p<0.001, highly significant

4: LVX+DAP versus LVX: p=0.009, significant

5: LVX+DAP versus DAP: p=0.26, not significant

357 Table 2. Amino acid substitutions in proteins and resulting minimal inhibitory concentration (MIC) changes of *S. aureus* MSSA 1112 mutants

Strain name	Strain and selection criteria	Amino acid (aa) substitutions and shifts in target proteins													MIC of the strains for (mg/L)			
		GrIA	GrIB	GyrB	MrpF	Clis2	AroK	Spa	YpdA	RpsU	RplU	FabI	PfIA	TlyC	DAP	LVX	CIP	VAN
MSSA 1112	Parent strain	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.25	0.25	≤1
MSSA 1112-2	MSSA 1112 mutant obtained after 12 cycles with increasing concentrations of DAP	-	-	-	P314L	A23V	Frameshift, addition of AA at position 290, change of aa at position 99 early stop codon at position 104	G22D	-	-	-	-	-	-	4	0.25	0.25	2
MSSA 1112-3	MSSA 1112 mutant obtained after 12 cycles with increasing concentration of LVX	S80F	-	W592R	-	-	-	-	-	-	-	-	-	-	0.5	2	4	≤1
MSSA 1112-4	MSSA 1112 mutant obtained after 12 cycles with ½ MIC LVX and increasing concentrations of DAP	P114L	-	-	-	-	-	-	Frameshift, AGTT deletion at position 1428, change of aa at position 477, early stop codon at position 487	Frameshift, insert of G at position 16, early stop codon at position 8	-	-	-	-	8	0.5	0.5	2
MSSA 1112-5	MSSA 1112 obtained after 12 cycles with ½ MIC DAP and increasing concentration of LVX	-	L440V	-	-	-	-	-	-	-	Frameshift, addition of CAAC at position 268, change of aa at position 92, early stop codon at position 95	D101Y	H143Y	Stop450W , extension of the protein with 27 aa	0.5	0.5	1	2

358 obtained after 12 repeated selective cycling with different concentrations of levofloxacin and daptomycin

359 CIP, ciprofloxacin; DAP, daptomycin; LVX, levofloxacin; VAN, vancomycin
360 Protein functions and known antibiotic target: GrIA (ParE), GrIB (ParC), DNA topoisomerase IV (fluoroquinolones); GyrB, DNA topoisomerase II
361 (fluoroquinolones); MrpF, phosphatidylglycerol lysyltransferase (daptomycin); Clis2, cardiolipin synthetase (daptomycin); AroK, shikimate kinase; Spa,

362 immunoglobulin G-binding protein A; YpdA, pyridine nucleotide-disulfide oxidoreductase; RpsU, 30S ribosomal protein S21 (daptomycin); RplU, 50S ribosomal
363 protein L21; FabI, enoyl-[acyl-carrier-protein] reductase; PflA, pyruvate formate-lyase-activating enzyme; TlyC, HlyC/CorC family transporter.