Antibiotics at Clinical Concentrations Show Limited Effectivity Against Acute and Chronic Intracellular *S. aureus* Infections in Osteocytes

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Running head: Antibiotic treatment of intra-osteocytic *S. aureus* infections

Abstract

Objectives: Case numbers of osteomyelitis are rising and chronic infections remain difficult to cure. While it is known that the major pathogen *Staphylococcus aureus* can persist intracellularly in osteocytes, the effectivity of antibiotics against this condition remains largely unknown. We sought to determine if current clinically utilised antibiotics were capable of clearing an intracellular osteocyte *S. aureus* infection.

Methods: Rifampicin, vancomycin, levofloxacin, ofloxacin, amoxicillin, oxacillin, doxycycline, linezolid, gentamicin and tigecycline were assessed for their MIC and minimum bactericidal concentrations (MBC) against 11 *S. aureus* clinical isolates and the reference strain ATCC 25923, at pH 5.0 and 7.2 to mimic lysosomal and cytoplasmic environments, respectively. Those antibiotics whose bone achievable concentration was commonly above their respective MICs for the strains tested were further assayed in a human osteocyte infection model under either acute or chronic conditions. Osteocyte-like cells were treated at 1, 4 and 10x the MIC for 1 and 7 days following infection (acute model), or after 14 days of infection (chronic model). The intracellular effectivity of each antibiotic was measured in terms of colony forming unit (CFU) reduction, small colony variant (SCV) formation and bacterial mRNA expression change.

Results: Only rifampicin, levofloxacin and linezolid reduced intracellular CFU numbers significantly in the acute model. The effect was larger after 7 days compared to 1 day of treatment. However, no treatment reduced the quantity of bacterial mRNA, nor prevented non-culturable bacteria from returning to a culturable state.

Discussion: These findings indicate that *S. aureus* adapts phenotypically during intracellular infection of osteocytes, adopting a reversible quiescent state which is protected against

antibiotics, even at 10x their MIC. Thus, new therapeutic approaches are necessary to cure *S. aureus* intracellular infections in osteocytes.

Keywords: Intracellular *S. aureus* infection; chronic osteomyelitis; intracellular antibiotic treatment

Introduction

Osteomyelitis is challenging to treat, and recurrent and chronic infections associated with treatment failure occur in up to 46% of cases [1, 2]. The most prevalent pathogen, *Staphylococcus aureus*, also has the highest treatment failure rate [2-4], and potentially contributing to this, *S. aureus* can persist intracellularly in phagosomes or the cytoplasm [5, 6] in immune cells and bone cells, including osteocytes [7].

The intracellular effectivity of antibiotics is difficult to predict and is often reduced due to host factors including intracellular metabolism, drug efflux, the low pH of certain intracellular compartments, as well as potential off-target effects on the pathogen clearance mechanisms of autophagy/xenophagy [8-11]. Bacterial factors also play a role, for example chronic osteomyelitis is associated with increased small colony variant (SCV) formation [4, 12, 13], resulting in higher antibiotic tolerance and immune protection [14, 15]. Furthermore, persister cells, a subpopulation of dormant bacteria with reduced metabolism, also confer antibiotic insensitivity [16]. Thus, to treat chronic osteomyelitis effectively, both intracellular location and phenotypic adaptation are potential factors to consider.

Osteocytes form a syncytial network throughout the bone and are the most abundant and long-lived resident population [17], theoretically providing an ideal niche for long-term infections, as has been reported with phenotypic adaption into SCV [12, 18, 19].

Here, we investigated the effectivity of clinically used antibiotics at concentrations predicted to be achievable in the bone in a human osteocyte-like intracellular infection model against *S. aureus*. Acute and chronic infections, short and long-term antibiotic treatments with antibiotics of different classes and mechanisms of action, with differing levels of evidence for effectivity [20], were investigated.

72

73 **Methods**

74 **Osteocyte-like Culture**

75 SaOS-2 cells (American Type Culture Collection (ATCC), Manassas, USA) were passaged
76 and differentiated to an osteocyte-like stage, as previously described [21]. The osteocyte-like
77 phenotype of the resulting cultures was confirmed by increased expression of phenotypic
78 markers *COL1A1*, *RUNX2*, *MEPE*, *BGLAP, DMP1* and *SOST* and *in vitro* mineralisation
79 (**Supp. Fig. 1**) [21]**.**

80 **Bacterial Isolates and Growth Conditions**

81 Institutional human research ethics clearance for this study was obtained (CALHN Approval
82 16633). Eleven *S. aureus* clinical isolates (CI) from the soft tissue wound or bone from patients
83 undergoing treatment for diabetic foot infection and the strain ATCC25923 were used. Bacteria
84 were grown in terrific broth (TB; Invitrogen, Waltham, USA), as previously described [21].
85 Viable bacterial concentrations were estimated from a CFU/ml *vs* $OD_{630\,nm}$ standard curve, then
86 validated by plating dilutions on nutrient broth (NB)-agar (Chem-supply, Gillman, Australia).

87 **MIC and Minimal Bactericidal Concentration (MBC)**

88 MICs for tigecycline, linezolid, oxacillin-sodium-salt-monohydrate, levofloxacin, ofloxacin
89 (Glentham Life Science, Corsham, UK), doxycycline-hydrate, vancomycin, amoxicillin and
90 rifampicin (Sigma-Aldrich, St Louis, USA) were determined with the micro-dilution method
91 in TB at pH 5.0 and pH 7.2 [22]. For MBC evaluation all clear wells and the first well showing
92 turbidity were spot-plated and colony growth was observed after 24 and 48 h. European
93 Committee on Antimicrobial Susceptibility Testing (EUCAST)-tables [23] were used to define
94 resistance.

95 **Intracellular Antibacterial Activity**

96 CI 8 was grown as described above and diluted to a multiplicity of infection (MOI) of 50 in
97 phosphate buffered saline (PBS). Intracellular infection was performed, as previously
98 described [19, 24]. Briefly, differentiated SaOS2 cells were exposed to the bacterial suspension
99 or sterile PBS for 2h. Following washing with PBS, all cells were re-incubated with 10 mg/l
100 lysostaphin for 2 h in antibiotic-free media and then treated with antibiotics at 1x, 4x, 10x their
101 respective MIC (i.e., 0.25, 1, 2.5 mg/l doxycycline-hydrate, 0.016, 0.064, 0.16 mg/l rifampicin,
102 0.0625, 0.25, 0.625 mg/l oxacillin, 1, 4, 10 mg/l levofloxacin and 2, 8, 20 mg/l linezolid) either
103 immediately or after 14 days. Cells were harvested for analysis after 1, 7, 15 and 21 days. To
104 test for outgrowth after antibiotic treatment in the chronic model, the 10x MIC treatments were
105 removed and the cells washed with PBS, before changing to an antibiotic- and lysostaphin-free
106 media. Media were spot-plated regularly and observed for changes in colour and cloudiness
107 daily. After 11 days, cells were washed and lysed to identify any intracellular CFU that did not
108 grow into the media. For CFU recovery, culture supernatants were tested for viable bacteria by
109 spot-plating. The host-cells were lysed in sterile water for 20 min, 1:10 serially diluted and
110 plated on NB-agar.

111 **Measurement of Gene Expression**

112 Cell supernatant was removed, and RNA isolated using TRI Reagent (Merck Life Science ,
113 VIC, Australia). After DNAse digestion, complementary DNA was synthesised with the iScript
114 gDNA clear cDNA Synthesis kit (BioRad, Hercules, USA), all as per manufacturer's
115 instructions. DNA was extracted with the Viagen DirectPCR® DNA Extraction System™ and
116 1% proteinase K (Viagen Biotech Inc, CA, USA) for 10 min at room temperature, 30 min at
117 55°C and 15 min at 85°C. DNA was digested with Ncol-HF (New England BioLabs, Notting

118 Hill, Australia). Real-time PCR reactions were performed using RT2 Forget-Me-Not™
119 EvaGreen qPCR Mastermix (Biotium, CA, USA) on a CFX Connect Real-Time-PCR-System
120 (BioRad). Measurement of effects on bacterial housekeeping gene levels in response to a 10x
121 MIC dose of each antibiotic was made against infected but untreated control or the
122 corresponding 1x MIC treatment. Oligonucleotide primer sequences are shown in
123 **Supplementary Table 1.**

124 **Statistics**

125 Differences in CFU, gene expression and proportion of SCV (%SCV) were analysed with
126 either a 1- or 2-way ANOVA with a Tuckey's multiple comparison or an unpaired t -Test (α =
127 0.05) using GraphPad Prism version 10.2.3 (GraphPad Software, La Jolla, USA). Graphs
128 herein show mean values ± standard deviation (SD).

129

130 **Results**

131 **MIC and MBC determination**

132 To determine which antibiotics could be effective at concentrations achievable in bone, we
133 determined their respective MIC and MBC against 11 CIs of *S. aureus* and ATCC 25923 at
134 both pH 5.0 and pH 7.2 (**Table 1**). All CIs were susceptible to vancomycin, linezolid,
135 gentamicin and tigecycline, while five strains were resistant to levofloxacin, ofloxacin and
136 oxacillin. The MBCs for some strains were at least 4-fold higher than the MICs for rifampicin,
137 oxacillin, doxycycline, linezolid and tigecycline, indicating a strong bacteriostatic effect. Some
138 antibiotics showed pH dependency, with levofloxacin, ofloxacin, doxycycline and tigecycline

139 being at least 4-times more effective at pH 7.2 than at pH 5.0 and rifampicin, oxacillin and
140 gentamicin being more effective at pH 5.0 than pH 7.2, consistent with previous studies [20].

141 The MIC and MBC values for each strain were compared to respective bone-achievable
142 concentrations [20]. Antibiotics that could reach a potentially therapeutic concentration in at
143 least 6 strains (i.e. >4x MIC [20]) were further tested in an intracellular infection model:
144 doxycycline, oxacillin, levofloxacin, rifampicin and linezolid.

145 **Infected Host-cell Survival**

146 To ensure that neither the bacterial infection itself, nor the antibiotic treatments affected host-
147 cell viability, we conducted live/dead staining at each time point and measured the *18S* rRNA
148 expression. There was no significant change due to the infection or any treatment at any time
149 point for either measure, as shown for levofloxacin (**Supp. Fig. 2**).

150 **Intracellular Antibacterial Activity**

151 We tested doxycycline, oxacillin, levofloxacin, rifampicin and linezolid for intracellular
152 antibacterial activity at 1, 4 and 10x their respective MIC for either 1 or 7 days and started
153 either immediately (acute model) or 15 days (chronic model) post-infection (**Fig. 1A**).

154 For the acute model, CFU recovery declined between 1 and 7 days post-infection for all
155 conditions (**Fig. 1B-F**). Beyond this, doxycycline had no effect on the number of intracellular
156 CFUs (**Fig. 1B, Table 2**). Oxacillin caused a significant, but clinically irrelevant CFU reduction
157 after 1 day of treatment at all concentrations, with no change after 7 days (**Fig. 1C, Table 2**).
158 Levofloxacin was ineffective after 1 day, however, 7 days of treatment resulted in a significant
159 and clinically relevant intracellular CFU-reduction of up to 2.98-log (**Fig. 1D, Table 2)**.
160 Rifampicin caused the highest significant dose-dependent CFU reduction after 1 day of up to
161 2.28-log, which increased up to 3.32-log after 7 days of treatment (**Fig. 1E, Table 2**). The

162 highest significant CFU reduction of 3.74-log was achieved after 7 days of treatment with
163 linezolid, however, linezolid had a significant but smaller impact after 1 day of treatment of
164 1.54-log (**Fig. 1F, Table 2**).

165 In the chronic infection model, few if any CFU were recovered, such that the change in CFU
166 number could not be statistically analysed (**Fig. 1B-F**).

167 To measure the effects of treatments on bacterial metabolism independent of effects on
168 culturability, we also examined bacterial mRNA expression, using *aroE* and *gyrB* as
169 representative genes. For the acute model, the impact of antibiotic treatments on bacterial
170 mRNA levels were not concordant with CFU recovery. Even at 10x MIC for 7 days no
171 antibiotic treatment reduced intracellular mRNA levels compared to the untreated control (**Fig.**
172 **1G**). Bacterial mRNA expression in the chronic model was similar to that of the acute model
173 and did not significantly change with any treatment throughout the time course (**Fig. 1G**).

174 To further examine potential effects of antibiotics on growth phenotype, the percentage of SCV
175 was recorded. Doxycycline, oxacillin, rifampicin and linezolid did not significantly impact the
176 %SCV (**Fig. 2B, C, E, F**). Levofloxacin caused a dose-dependent increase in the %SCV up to
177 28.21% (**Fig. 2D**).

178 **Bacterial Resuscitation**

179 To test the ability of antibiotic-treated cultures displaying non-culturable infections to give rise
180 to a culturable infection, a bacterial outgrowth assay was performed. Following robust
181 treatment of intracellular bacteria with a 3 day 10x MIC antibiotic regimen, antibiotic pressure
182 was removed. As shown in **Figure 3,** bacterial resuscitation occurred in untreated control
183 cultures after 7 days, with approximately 60% of wells showing outgrowth. Outgrowth
184 occurrence was similar for doxycycline, levofloxacin and linezolid, while outgrowth was

185 evident earlier, at 4 days, for oxacillin-treated cultures. Most wells containing culturable
186 extracellular bacteria also had culturable bacteria in the host cell lysate at the experimental end.
187 However, rifampicin treatment only yielded culturable bacteria from the host cell lysate (**Fig.**
188 **3**).

189 Intracellular bacterial DNA levels tended to mirror the change in bacterial outgrowth, clearly
190 increasing between days 1-11 post-treatment, consistent with increased bacterial replication,
191 with the exception of rifampicin-treated cultures which displayed stable bacterial levels (**Fig.**
192 **3**).

193

194 **Discussion**

195 This study highlights an important limitation of currently available antibiotics, in that despite
196 bone penetrance, while culturability may be impaired, intracellular bacterial viability and
197 activity (DNA and mRNA levels) persist, indicating a switch to a non-culturable but
198 nevertheless potentially virulent phenotype. This serves as further evidence that phenotype
199 switching to low- or non-culturable phenotypes is an important and emerging aspect of
200 intracellular infections in osteomyelitis and is likely to contribute to antimicrobial treatment
201 failure and persistent infection [11, 12, 25].

202 Rifampicin is among the most demonstrably effective antibiotics against intracellular *S. aureus*
203 [20]. This was confirmed here as it demonstrated a 2-8-fold higher sensitivity at pH 5.0
204 compared to pH 7.2, indicating high lysosomal activity, and up to a 3.7-log CFU reduction in
205 the osteocyte model. Furthermore, rifampicin was the only treatment that did not yield
206 culturable bacteria in the media after antibiotic removal and the only antibiotic that inhibited

207 bacterial population growth following the cessation of treatment, as evidenced by the lack of
208 increase in bacterial DNA levels, at least within the time frame of the analysis.

209 Linezolid was the only protein-biosynthesis-inhibitor tested that effectively reduced
210 intracellular CFU numbers up to a 3.74-log, in particular after a 7 day treatment. However, it
211 appeared to impact the growth phenotype, as apparent persister cells readily reverted and
212 escaped after antibiotic removal.

213 The fluoroquinolones ofloxacin and levofloxacin showed an approximately 10-fold reduced
214 susceptibility at pH 5.0 and six CIs were resistant [23]. This resulted in a higher MIC than the
215 3 mg/l achievable in bone for ofloxacin, however, for levofloxacin, when susceptible, the MIC
216 was between 0.5-5 mg/l at both pH levels tested, a range that easily falls below the maximum
217 concentration of 10 mg/l achievable in bone [20]. Levofloxacin reduced CFU numbers up to
218 2.98-log after 7 days of treatment. Still, levofloxacin failed to reduce the bacterial mRNA levels
219 at any stage and showed a significant dose-dependent increase in %SCV after 7 days of
220 treatment. Therefore, fluoroquinolones might be effective in reducing intracellular CFU
221 numbers if the bacterial strain is sensitive, however, they also might induce phenotypic
222 switches to less antibiotic and immune-susceptible phenotypes.

223 Doxycycline showed a strongly strain-dependent MIC, lower than the reported maximum bone
224 concentration up to 3 mg/l. Nevertheless, it proved to be ineffective in the osteocyte model.

225 The three cell wall inhibitors (oxacillin, amoxicillin and vancomycin) were found to be
226 ineffective in reducing intracellular bacterial numbers. Oxacillin was much more effective at
227 pH 5.0 than at pH 7.2 and at a much lower concentration than the maximum bone level of 4-
228 10 mg/l [20]; however, this was ineffective in the osteocyte model. Gentamicin is often
229 described as only being effective against extracellular pathogens [26]. Nonetheless, the

230 cell:plasma ratio measured in macrophages between 4.4 and 6.8 is comparable to other
231 antibiotics with intracellular activity, such as linezolid and oxacillin [8, 9]. We observed a 2-
232 to 8-fold reduced effectivity at pH 5.0 compared to pH 7.2, which might contribute to the low
233 intracellular activity of 1-3-log reported in bone cells [13, 20], when given at concentrations
234 that exceed those that can be expected in the bone due to poor bone penetration [20]. Therefore,
235 gentamicin does not seem to be a suitable candidate to treat chronic osteomyelitis. Tigecycline
236 and vancomycin showed higher MICs than can be achieved in the bone and were therefore not
237 tested in the osteocyte model.

238 Limitations of this study include the number of antibiotics tested and the mono-therapeutic
239 approach. Clinically utilised antibiotics were selected to represent various mechanisms of
240 action and included those that were predicted to be most effective [20]. Further, even though
241 the MIC/MBC were tested in multiple CIs, for feasibility purposes, the intracellular infection
242 assay was only conducted with one strain. However, this isolate, an actual osteomyelitis strain,
243 brings further clinical relevance to the findings. The purpose of the model utilised was to
244 facilitate high-throughput testing of antimicrobial effectivity against intracellular *S. aureus* in
245 a human osteocyte-like context. To achieve reproducibility, we used an osteocyte-like cell line
246 model rather than primary cells [12], which might behave differently. However, osteogenically
247 differentiated SaOS-2 is a validated human osteocyte-like model for infection experiments,
248 shown to possess key properties of primary cells [19, 21, 27], as also verified here.

249 In conclusion, the most effective antibiotics at clinically-relevant concentrations for reducing
250 intracellular CFUs in a human osteocyte-like cell model were rifampicin, levofloxacin and
251 linezolid. Treatment duration has a significant impact on the effectivity of these antibiotics.
252 However, treatments that were effective in reducing bacterial CFU numbers, did not eradicate
253 the bacteria, as intracellular bacterial DNA and mRNA remained detectable and bacteria may

switch to a non-culturable state and regrow once antibiotic pressure is removed. In this respect, antibiotics treatments serve as agents for selecting persisting bacterial populations, rather than curing the intracellular infection. The impact of unsuccessful antimicrobial treatment of intracellular infection on the induction of antibiotic resistance is yet to be investigated. The model described herein could be used to examine strain-specific antimicrobial effectivity for the purposes of optimising treatment of osteomyelitis, and to test new or emerging drugs in this context.

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268

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270 None of the authors has any conflicts of interest, financial or otherwise, to disclose.

271

272 **Author Contributions**

273 ARZ project conception, performed experiments, manuscript draft

274 DY, NJG, KR, SPK experimental support, manuscript development

275 LBG, RN manuscript development

276 GJA, project conception, manuscript development, communicating author.

277

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349

350 **Figure legends**

351 **Figure 1:** Intracellular bacterial culturability in acute and chronic *S. aureus* infection models.
352 A) Schematic of the experimental design of the osteocyte infection and antibiotic treatment
353 model. Upon the introduction of *S. aureus* infection to SaOS2-OY cells, antibiotic (AB)
354 treatments were provided to cultures either immediately after the infection on day 0 for the
355 acute model or 14 days post-infection for the chronic model. Recovered CFU from acute
356 (sampled at days 1 and 7) and chronic (sampled at days 15 and 21) models under antibiotic
357 treatments of B) doxycycline, C) oxacillin, D) levofloxacin, E) rifampicin and F) linezolid, at
358 the doses of 0, 1, 4 and 10x the respective MIC; G) Fold-change in mRNA levels of two
359 bacterial genes, *aroE* and *gyrB*, among individual antibiotic treatment groups compared to
360 expression in the untreated control, on days 7 and 21 for acute and chronic models, respectively.

361

362 **Figure 2:** Effects of antibiotics on colony phenotype of *S. aureus* recovered from an
363 intracellular infection in osteocytes. A) Example image showing small colony variants (SCV;
364 *red arrows*), defined here as a colony after 24 hours of culture on nutrient agar measuring <0.5
365 mm; B-F) The %SCV from host-cell lysates was determined after 1 and 7 days of treatment
366 with B) doxycycline, C) oxacillin, D) levofloxacin, E) rifampicin and F) linezolid, at 1x, 4x
367 and 10x their respective MIC ($n \geq 3$). Significant differences are indicated by * $p < 0.05$.

368

369 **Figure 3:** Outgrowth of intracellular *S. aureus* from a non-culturable state. SaOS2-OY cells
370 were infected with *S. aureus* and intracellular bacteria *were* confirmed non-culturable by 14
371 days post-infection. All cells were then treated for 3 days with either antibiotic-free media or
372 with antibiotics at their respective 10x MIC. For all treatment groups (A: no antibiotic; B:

373 doxycycline; C: oxacillin; D: levofloxacin; E: Rifampicin; F: Linezolid), cell culture media
374 were tested for extracellular bacteria culturability from potential outgrowth on days 4, 7 and
375 11 (n = 32 wells per condition, line graphs on left Y-axis). Cell lysates from the experiment
376 endpoint (day 11) were tested for intracellular bacterial culturability (n = 28 wells per
377 condition, bar graphs on left Y-axis, hatched pattern indicates growth). The number of wells
378 with or without outgrowth are shown adjacent. Total DNA isolated from cell lysates on days 1
379 and 11 was measured for intracellular bacterial genome levels by qPCR (n = 4 wells per
380 condition; dot graphs on right Y-axis).

381

382 **Supplementary Figure 1:** Osteocytic differentiation of SaOS-2 cells. A-F) Osteogenic gene
383 expression of osteocyte markers during differentiation relative to *18S* rRNA levels ($n = 4$). *p
384 < 0.05, ***$p < 0.001$ and ***$p < 0.0001$. Gene expression was normalised to that of the
385 housekeeping gene *18S* and calculated with the $2^{-\Delta Ct}$ method. G) Von Kossa stain for phosphate
386 and Alizarin Red stain for calcium to observe mineralisation of osteocytes after 7 and 14 days
387 of differentiation ($n = 4$).

388

389 **Supplementary Figure 2:** Survival of host-cells during intracellular infection. A) Levels of
390 *18S* rRNA over time in infected control, uninfected control or treatment with levofloxacin (as
391 a representative example for all treatments; $n = 4$). B) Live/dead staining of infected and
392 uninfected controls. Cells were cultured as described above, on Cell Imaging Plates
393 (Eppendorf, Germany). Immediately before the infection or 1, 7, 15 and 21 days after the
394 infection the media of infected and uninfected wells were incubated in the dark at 37°C/ 5%
395 CO_2/ 1% O_2 with eBioscience TM Calcein Violet 450 AM Viability Dye (live, Invitrogen) and

396 Ethidium Homodimer III (dead, Biotium) for 30 min. Confocal images were then taken with
397 an Olympus FV3000 confocal microscope (Olympus, Tokyo, Japan) and processed with
398 ImageJ software (Bethesda, USA) to obtain relative intensities of live/dead stained cells. Data
399 shown as means ± standard error of the mean for 4 biological replicates, in at least 3 regions of
400 interest per well.

401

402 **Table 1:** Antimicrobial susceptibility of *S. aureus* and antimicrobial concentrations.

Antibiotic	pH		Clinical Isolates											ATCC	Cmax bone
			1	2	3	4	5	6	7	8	9	10	11	25923	
Rifampicin	5.0	MIC	0.008	0.004	0.008	0.008		0.00125	0.002	0.008	0.008		0.005	<0.004	1.3-6.5
		MBC	0.032	0.008	0.008	0.016		0.002	0.005	0.016	0.016		>0.064	0.008	
	7.2	MIC	0.032	0.016	0.016	0.016	0.008	0.005	0.016	0.016	0.016	0.005	>0.064	0.008	
		MBC	0.064	0.032	0.032	0.032	0.016	0.8	>0.064	>0.064	>0.064	0.064	>0.064	0.016	
Vancomycin	5.0	MIC	2	1	1	1		1	2	1	1		1	2	1.1-6
		MBC	2	2	4	4		8	4	2	4		8	4	
	7.2	MIC	1	1	2	1	2	1	1	1	1	0.5	1	2	
		MBC	2	1	1	1	2	2	2	1	8	0.5	8	4	
Levofloxacin	5.0	MIC	5	>500	500	500		5	5	5	5		10	5	4.6-10
		MBC	10	>500	>500	>500		10	10	50	10		50	5	
	7.2	MIC	0.5	50	50	50	100	0.5	0.5	0.5	0.5	50	1	0.5	
		MBC	1	50	50	50	>500	1	1	10	0.5	>500	50	0.5	

Ofloxacin	5.0	MIC	10	500	500	100		10	10	5	5		50	5	3
		MBC	10	>500	>500	>500		15	50	10	10		100	10	
	7.2	MIC	0.5	100	100	100	100	5	1	0.5	0.5	100	1	0.5	
		MBC	1	>500	>500	>500	>500	5	5	5	1	>500	50	1	
Amoxicillin	5.0	MIC	0.25	1	1	2		0.25	0.25	1	1		0.25	0.125	27-37
		MBC	0.5	>2	>2	>2		2	0.25	2	2		>2	0.25	
	7.2	MIC	0.0625	>2	>2	>2	1	0.125	0.0625	>2	>2	1	0.5	0.0625	
		MBC	0.0625	>2	>2	>2	2	0.25	0.25			2	>2	0.25	
Oxacillin	5.0	MIC	0.0625	0.0625	0.03125	0.125		0.03125	0.03125	0.03125	0.03125		2.5	0.0625	4-10
		MBC	0.625	0.25	0.125	1		0.0625	0.0625	0.0625	0.0625		>80	0.125	
	7.2	MIC	0.0625	0.5	1	2	>2	0.25	0.125	0.25	0.25	20	20	0.25	
		MBC	0.5	>2	>2	>2	>2	1	0.25	2	0.25	40	>80	0.5	
Doxycycline	5.0	MIC	0.125	0.25	0.125	0.25		0.25	0.25	0.25	0.25		16	0.0625	3
		MBC	2	0.25	1	8		16	16	2	0.5		>16	2	
	7.2	MIC	0.125	<0.03125	<0.03125	<0.03125	8	0.125	0.25	0.125	0.5	8	8	0.0625	

	MBC	2	0.0625	0.125	8	>16	>8	8	1	2	16	>16	0.25	
Linezolid	5.0 MIC	1	2	2	2		4	4	2	2		2	2	6.3-9.1
	5.0 MBC	64	64	16	64		64	32	8	32		64	16	
	7.2 MIC	2	1	1	1	1	4	4	2	2	<0.5	1	2	
	7.2 MBC	16	2	1	32	4	32	64	16	8	<0.5	64	16	
Gentamicin	5.0 MIC	32	>64	>64	>64		>64	>64	64	>64		>64	>64	Detectable
	5.0 MBC	>64	>64	>64	>64		>64	>64	>64	>64		>64	32	
	7.2 MIC	2	4	4	4	8	16	16	4	32	16	64	8	
	7.2 MBC	16	16	8	16	16	32	32	32	32	64	>64	8	
Tigecycline	5.0 MIC	4	8	8	8		8	8	8	8		0.125	4	0.3
	5.0 MBC	>8	8	8	8		>8	8	7	>8		4	4	
	7.2 MIC	0.125	0.125	0.125	0.125	0.25	0.125	0.125	0.125	0.125	0.125	2	0.0625	
	7.2 MBC	2	0.5	1	1	2	2	2	0.5	0.5	0.5	8	2	

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for antibiotics tested against *S. aureus* strains at pH 5.0 and pH 7.2. Values shown are in µg/ml. Cmax bone = the maximal concentration achievable in bone [20].

405 **Table 2**: Antimicrobial effect on colony forming units (CFU) in an acute intra-osteocytic *S.*
406 *aureus* infection.

	Treatment	1-day treatment		7- day treatment	
		Mean ± SD	*Log Red*	*Mean ± SD*	*Log Red*
Doxycycline	Infected CTRL	866667 ± 325167		36000 ± 10000	
	1 x MIC	74667 ± 227450	0.06	47333 ± 15011	-0.12
	4 x MIC	753333 ± 133167	0.06	79333 ± 22301	-0.34
	10 x MIC	473333 ± 46188	0.26	36000 ± 19698	0
Oxacillin	Infected CTRL	20400 ± 7292		1100 ± 698	
	1 x MIC	5355 ± 4168	0.58*	455 ± 200	0.38
	4 x MIC	5725 ± 1612	0.55*	390 ± 125	0.45
	10 x MIC	3240 ± 817	0.80*	122 ± 63	0.96
Levofloxacin	Infected CTRL	730000 ± 728080		13933 ± 4291	
	1 x MIC	264667 ± 28095	0.44	840 ± 640	1.22***
	4 x MIC	534667 ± 715476	0.14	103 ± 2	2.13***
	10 x MIC	66667 ± 14742	1.04	15 ± 1	2.98***
Rifampicin	Infected CTRL	100000 ± 59025		2080 ± 413	
	1 x MIC	11933 ± 5368	0.92***	33 ± 30	1.80****
	4 x MIC	1620 ± 851	1.79***	43 ± 75	1.68****
	10 x MIC	520 ± 320	2.28***	0	3.32****
Linezolid	Infected CTRL	19867 ± 3521		5490 ± 2079	
	1 x MIC	13333 ± 4407	0.17*	498 ± 271	1.04**
	4 x MIC	8777 ± 7479	0.35****	7 ± 2	2.87***
	10 x MIC	573 ± 628	1.54****	0	3.74***

407 Logarithmic CFU reduction in an acute infection model of SaOS-2 osteocyte of each antibiotic
408 at 1, 4 and 10x MIC after 1 and 7 days of treatment. Asterisks indicates significant CFU log

409 reduction (Log Red) compared to an untreated control with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

410 and ***$p < 0.0001$.

411

412 **Supplementary Table 1:** Oligonucleotide primer sequences for real time PCR.

GENE	*FORWARD PRIMER*	*REVERSE PRIMER*
18S [1]	GCGTTGATTAAGTCCCTGCC	CACCTACGGAAACCTTGTTACGAC
AROE [5]	CTATCCACTTGCCATCTTTTAT	ATGGCTTTAATATCACAATTCC
GYRB [5]	AATTGAAGCAGGCTATGTGT	ATAGACCATTTTGGTGTTGG
MEPE [4]	AGATTCTCAAAGATGCGAGTTTTC	CCTCTGCTCTTCCACACAGC
RUNX2 [4]	CCTTCAAGGTGGTAGCCCCT	GGTGAAACTCTTGCCTCGTC
BGLAP [3]	ATGAGAGCCCTCACACTCCTCG	GTCAGCCAACTCGTCACAGTCC
SOST [2]	ACCGGAGCTGGAGAACAACA	GCTGTACTCGGACACGTCTT
DMP1 [2]	GATCAGCATCCTGCTCATGTT	AGCCAAATGACCCTTCCATTC

413

414 1. Yang, D., et al., *Novel insights into Staphylococcus aureus deep bone infections: The*
415 *involvement of osteocytes.* mBio, 2018. **9**(2): p. 1-10.
416 2. Atkins, G.J., et al., *The induction of a catabolic phenotype in human primary*
417 *osteoblasts and osteocytes by polyethylene particles.* Biomaterials, 2009. **30**(22): p.
418 3672-3681.
419 3. Atkins, G.J., et al., *Metabolism of vitamin D3 in human osteoblasts: evidence for*
420 *autocrine and paracrine activities of 1α, 25-dihydroxyvitamin D3.* Bone, 2007. **40**(6):
421 p. 1517-1528.

422 4. Zelmer, A.R., et al., *Saos-2 cells cultured under hypoxia rapidly differentiate to an*
423 *osteocyte-like stage and support intracellular infection by Staphylococcus aureus.*
424 Physiological Reports, 2023. **11**(21): p. e15851.

425 5. Kalinka, J., et al., *Staphylococcus aureus isolates from chronic osteomyelitis are*
426 *characterized by high host cell invasion and intracellular adaptation, but still induce*
427 *inflammation.* International Journal of Medical Microbiology, 2014. **304**(8): p. 1038-
428 1049.

429

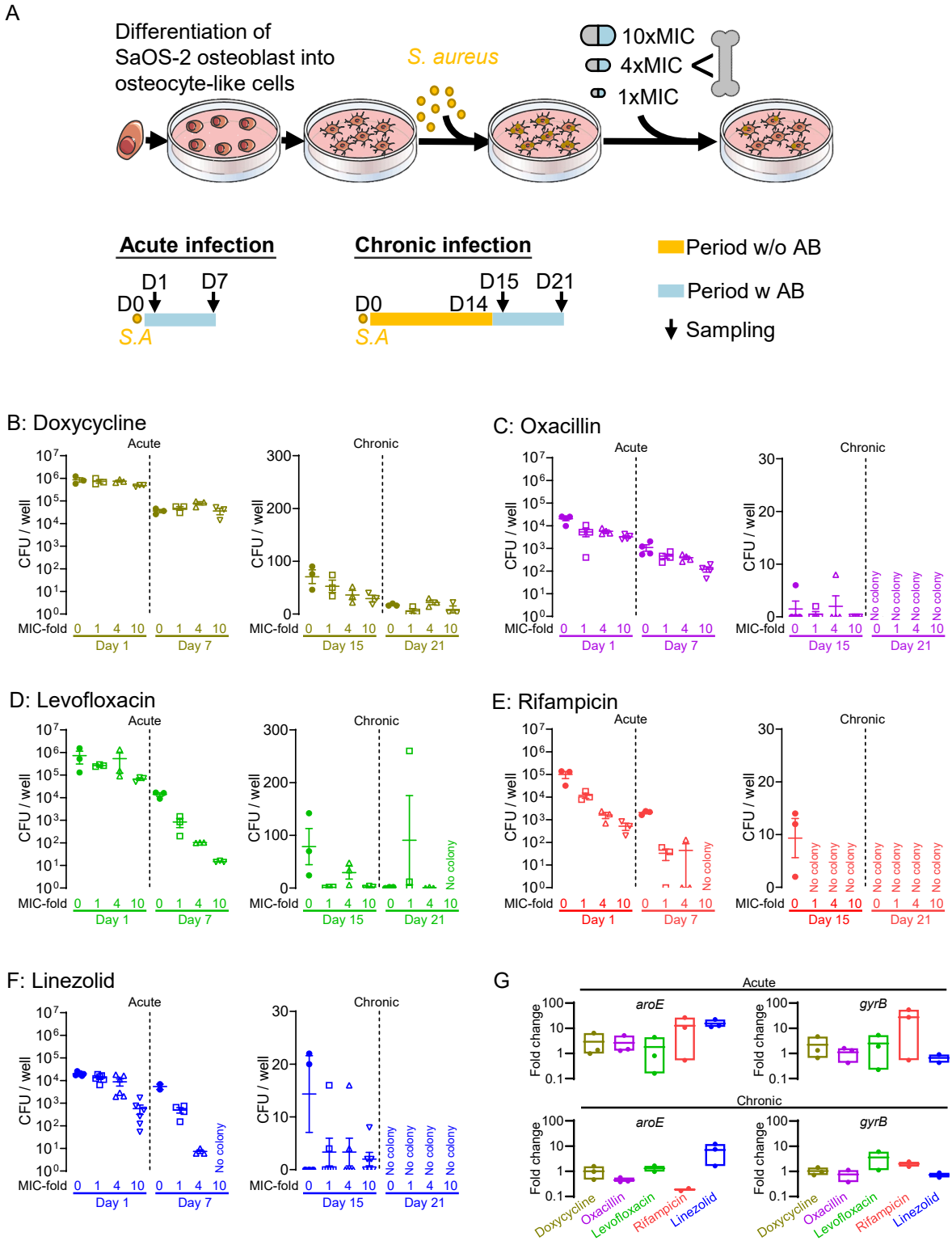
A
Differentiation of SaOS-2 osteoblast into osteocyte-like cells
S. aureus
10xMIC
4xMIC
1xMIC
Acute infection
D0
D1
D7
S.A
Chronic infection
D0
D14
D15
D21
S.A
Period w/o AB
Period w AB
Sampling
B: Doxycycline
C: Oxacillin
D: Levofloxacin
E: Rifampicin
F: Linezolid
Acute
Chronic
CFU / well
MIC-fold
Day 1
Day 7
Day 15
Day 21
No colony
G
aroE
gyrB
Fold change
Doxycycline
Oxacillin
Levofloxacin
Rifampicin
Linezolid

Figure 1

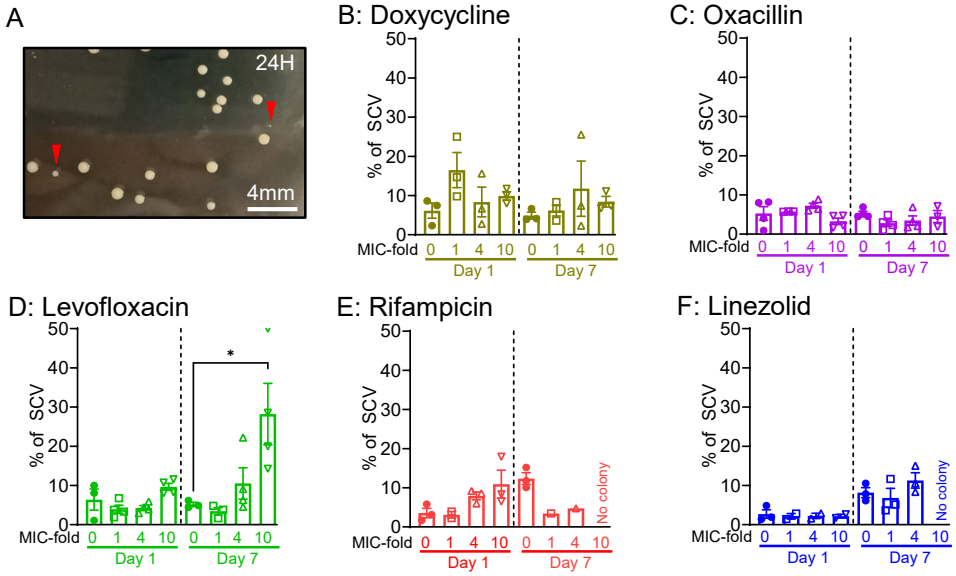
A
24H
4mm
B: Doxycycline
C: Oxacillin
D: Levofloxacin
E: Rifampicin
F: Linezolid
% of SCV
MIC-fold
0 1 4 10
Day 1
Day 7
*
No colony

Figure 2

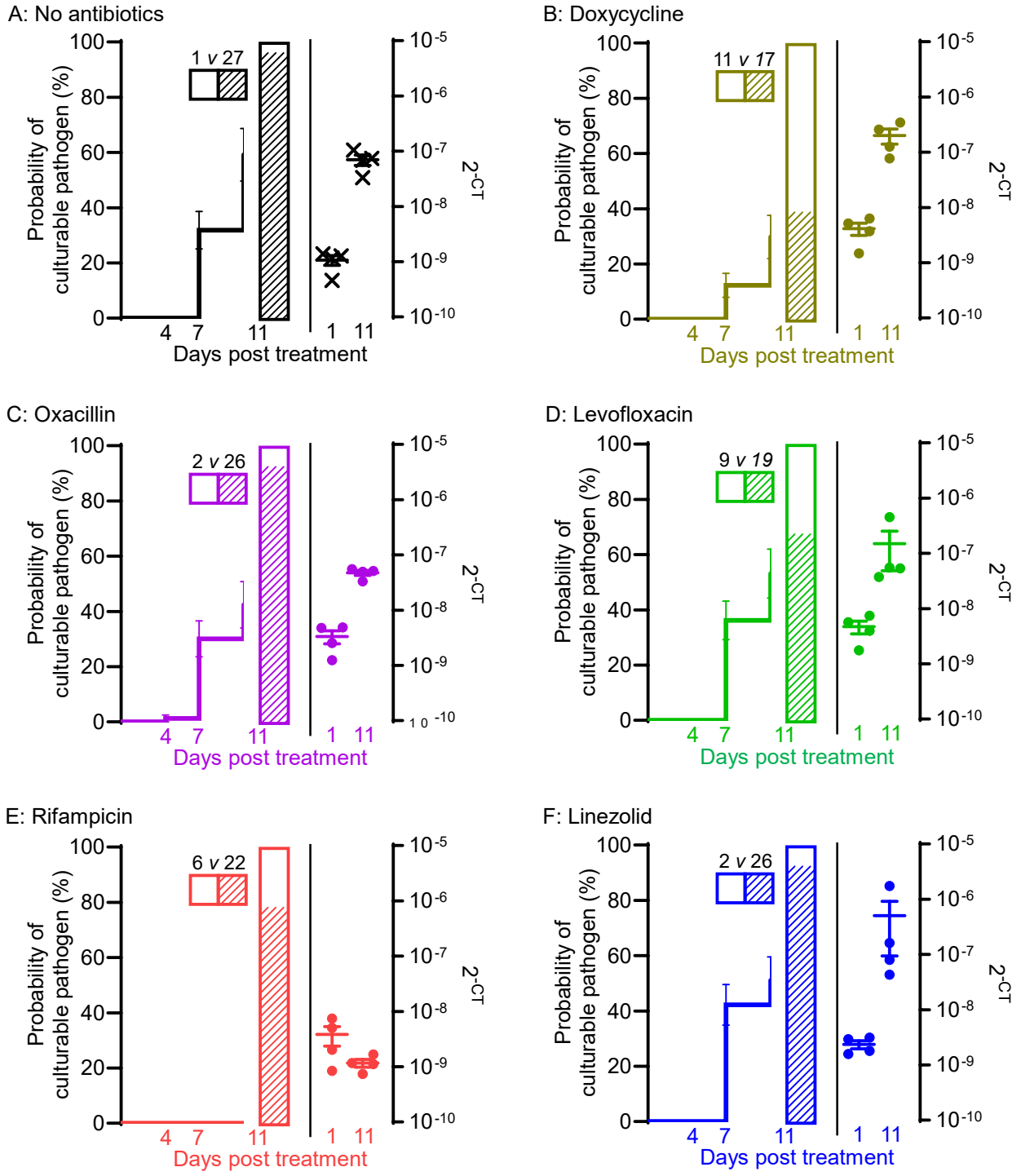
A: No antibiotics
1 v 27
B: Doxycycline
11 v 17
C: Oxacillin
2 v 26
D: Levofloxacin
9 v 19
E: Rifampicin
6 v 22
F: Linezolid
2 v 26
Probability of culturable pathogen (%)
Days post treatment
2-CT
0
20
40
60
80
100
4
7
11
1
11
10-5
10-6
10-7
10-8
10-9
10-10

Figure 3