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Strain-to-strain variability among *Staphylococcus aureus* causing prosthetic joint infection drives heterogeneity in response to levofloxacin and rifampicin

María Ángeles Meléndez-Carmona [1], Mikel Mancheño-Losa [2]*, Albert Ruiz-Sorribas[3], Irene Muñoz-Gallego [1], Esther Viedma[1], Fernando Chaves[1], Françoise Van Bambeke [3] and Jaime Lora-Tamayo [2,4]

[1]Department of Clinical Microbiology, Hospital Universitario 12 de Octubre, Instituto de Investigación Biomédica 'i+12' Hospital 12 de Octubre, Madrid, Spain; [2]Department of Internal Medicine, Hospital Universitario 12 de Octubre, Instituto de Investigación Biomédica 'i+12' Hospital 12 de Octubre, Madrid, Spain; [3]Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium; [4]CIBER Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain

*Corresponding author. E-mail: mikel.mancheno@gmail.com

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Introduction: Levofloxacin and rifampicin are the preferred treatment for prosthetic joint infection (PJI) caused by *Staphylococcus aureus*, especially when managed with implant retention (DAIR). However, a significant variability of success has been reported, which could be related to intrinsic characteristics of the microorganism. Our aim was to evaluate the variability in the anti-biofilm response to levofloxacin and rifampicin in a clinical collection of *S. aureus*.

Material and methods: Eleven levofloxacin- and rifampicin-susceptible *S. aureus* isolates causing PJI managed with DAIR were included. Levofloxacin, rifampicin and levofloxacin + rifampicin were tested in an *in vitro* static biofilm model in microtitre plates, where 48 h biofilms were challenged with antimicrobials during 24 h. Additionally, two genetically similar strains were tested in the CDC Biofilm Reactor, where 48 h biofilms were treated during 56 h. Antimicrobial activity was assessed by viable biofilm-embedded cells recount, and by crystal violet staining.

Results: All antimicrobial regimens showed significant anti-biofilm activity, but a notable scattering in the response was observed across all strains (inter-strain coefficient of variation for levofloxacin, rifampicin and levofloxacin + rifampicin of 22.8%, 35.8% and 34.5%, respectively). This variability was tempered with the combination regimen when tested in the biofilm reactor. No correlation was observed between the minimal biofilm eradicative concentration and the antimicrobial activity. Recurrent *S. aureus* isolates exhibited higher biofilm-forming ability compared with strains from resolved infections (7.6 $\log_{10}$ cfu/cm^2±0.50 versus 9.0 $\log_{10}$ cfu±0.07).

Conclusions: Significant variability may be expected in response to levofloxacin and rifampicin among biofilm-embedded *S. aureus*. A response in the lower range, together with other factors of bad prognosis, could be responsible of treatment failure.

Introduction

Staphylococcus aureus remains a major cause of prosthetic joint infection (PJI). In this context, common antibiotic susceptibility tests do not reliably predict the antimicrobial response that is generally expected of infections caused by planktonic bacteria.[1]

Despite these difficulties, the combination of rifampicin plus a fluoroquinolone (e.g. levofloxacin) is currently the treatment of choice for acute staphylococcal PJI managed with debridement, antibiotics and implant retention (DAIR).[2] Unfortunately, the heterogeneity observed among studies addressing the efficacy of DAIR yields quite a wide range of failure rate.[3,4] While this probably reflects the diversity among studies, it may also be caused by the confluence of multiple host parameters,[5] and also other important variables related to the causative staphylococcal strain, its genotypic background and virulence intrinsic characteristics.[6,7]

Our group recently tested the activity of both rifampicin and levofloxacin against intracellular strains of *S. aureus* responsible for PJI.[8] While all antimicrobials were active, a striking heterogeneity was observed between specific strains.

To complement these findings, we conducted a study to assess variability in response to levofloxacin and rifampicin among

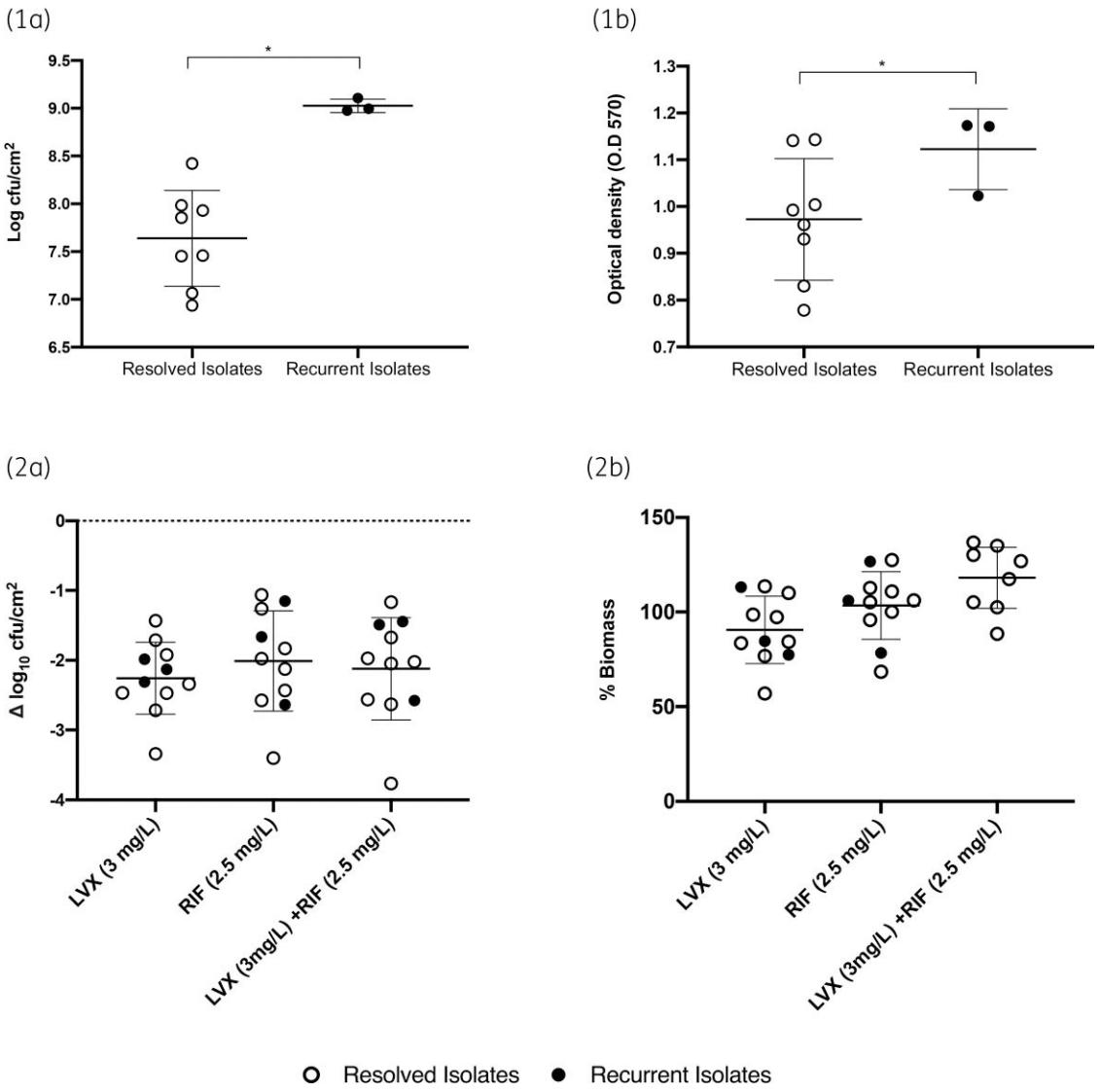

Figure 1. Biofilm-forming ability of *S. aureus* and efficacy of antimicrobials against biofilms (static model). Panel 1: biofilms were incubated during 48 h in the absence of drugs. The ordinate shows (1a) cfu counting (expressed in $\log_{10}$ cfu/cm^2) and (1b) crystal violet absorbance (measured at 570 nm). Panel 2: 48 h biofilms were incubated with levofloxacin (LVX, 3 mg/L) and rifampicin (RIF, 2.5 mg/L), alone and in combination, during 24 h. Results are expressed as (2a) the reduction of $\log_{10}$ cfu/cm^2 compared with control (Δ $\log_{10}$ cfu/cm^2). (2b) Biomass [assessed by crystal violet; expressed as the percentage of the value measured in control conditions (no antibiotics added)]. White circles indicate resolved cases, whereas black circles denote recurrent cases. Each dot represents the result of three replicates of each isolate. Bar denotes mean ± SD of the isolates grouped. Statistical analysis was performed by using unpaired Student's *t*-test and by ANOVA with Tukey's *post hoc* test per treatment. *$P < 0.05$.

a wide spread of staphylococcal strains causing PJI in two experimental models of non-intracellular staphylococcal biofilm.

Material and methods

Further details are included in the Supplementary data, available at *JAC* Online.

Bacterial isolates

The *S. aureus* isolates were obtained from a prospective multicentre study.[6] We selected 11 strains responsible for acute PJI cases managed with DAIR and rifampicin+levofloxacin (Table S1). Three cases (27%) eventually failed due to the same *S. aureus* (confirmed by PFGE) that caused the original infection (recurrent isolates), and eight were considered cured (resolved isolates) after a median follow-up of 36.5 months (IQR 13.8–17.7).

Antimicrobials and susceptibility testing

Levofloxacin and rifampicin drug powders were reconstituted following CLSI guidelines.[9] Susceptibility was measured by the Etest method following EUCAST guidelines.[10] Biofilm susceptibility was also performed by determining the minimum biofilm eradication concentrations (MBEC) and minimum biofilm inhibitory concentration (MBIC) as previously described.[1]

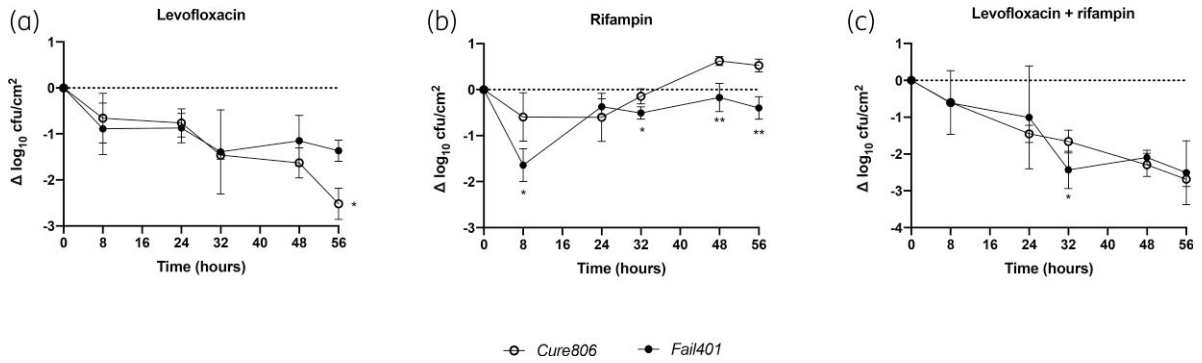

Figure 2. Bacterial killing of biofilm-embedded cells from coupons treated with monotherapies of (a) levofloxacin (LVX), (b) rifampicin (RIF), and (c) LVX-RIF combination in the CDC biofilm reactor. Experimental concentration for LVX and RIF was 3 and 2.5 mg/L, respectively. Results are expressed using the $\log_{10}$ change method from time 0 to each timepoint (mean ± SD). Biofilm bacterial densities at the beginning of the experiment were 5.4 ± 0.4 $\log_{10}$ cfu/cm^2 and 6.5 ± 0.08 $\log_{10}$ cfu/cm^2 ($P = 0.001$) for *Cure*806 and *Fail*401, respectively, and they remained stable throughout the following 56 h when no antibiotics were administered. Shapiro–Wilks test was tested for normality and the unpaired Student's *t*-test or Mann–Whitney *U*-test were used to compare differences between two groups. *$P < 0.05$; **$P < 0.01$.

Static in vitro biofilm model

Biofilms were obtained by growing a suspension of *S. aureus* for 48 h in a 12-well plate containing titanium-alloy (Ti6Al4V) disc coupons in RPMI-1640 medium supplemented with KH_2PO_4 50 mM + Na_2HPO_4 74.1 mM+1% glucose (pH 7, 37°C) as previously described (Figure S1).[11] After 48 h, mature biofilms were exposed to antibiotics for 24 h, namely levofloxacin, rifampicin, alone and in combination at concentrations that would be expected in human cortical bone at standard doses (levofloxacin, 750 mg/day; rifampicin, 600 mg/day): 3 mg/L and 2.5 mg/L, respectively (Table S2).[12] Biofilm-embedded bacteria were recovered by two alternating cycles of vortexing and sonicating (40 Hz), then serially diluted and plated to allow cfu counting after overnight incubation at 37°C. In addition, biofilm biomass was quantified using modified crystal violet staining.[11] The biomass was expressed as a percentage of the absorbance value measured under control conditions (no antibiotics added) at 570 nm.

Dynamic in vitro pharmacokinetics/pharmacodynamics (PK/PD) biofilm model

Biofilms were grown on 24 titanium-alloy disc coupons using the CDC biofilm reactor system (Biosurface Technologies Corp., Bozeman, MT, USA), as described elsewhere.[13] Briefly, biofilms were formed after a 48 h conditioning phase, which included 24 h of batch culture [TSB (Tryptic Soy Broth) with 1% glucose] followed by 24 h of continuous flow of medium (20% TSB), mixed by a stir bar at 130 rpm, and at 37°C. The therapeutic phase was then started (time 0) and antibiotics were administered as bolus every 24 h, with the same C_{max} as in the previous model. During this phase, 20% TSB was pumped at a rate that simulated the half-life of each antibiotic (Table S2). Three coupons were collected aseptically at 0, 8, 24, 32, 48 and 56 h. Biofilm-embedded bacteria were recovered as previously described.[13]

Appropriate levofloxacin and rifampicin concentrations in the reactor throughout the experiment were confirmed by the bioassay method in Difco Antibiotic Medium No. 1 (Becton Dickinson), using *Escherichia coli* ATCC 21922 and *Staphylococcus epidermidis* ATCC 27626, respectively.

The emergence of resistant strains at all timepoints was examined using agar plates with levofloxacin and rifampicin concentrations of 1 mg/L.

Statistical analysis

Statistical analysis and graphs were done using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Unpaired Student's *t*-test or Mann–Whitney *U*-test and the analysis of variance (ANOVA) with Tukey's *post hoc* test per treatment were used to compare differences between groups. *P* values of <0.05 were considered to be significant.

Results

Static biofilm model

The strains studied exhibited heterogeneous biofilm formation with interstrain variability of 9.6% (Figure 1). Recurrent *S. aureus* isolates showed biofilms with higher cell densities (9.0 ± 0.07 $\log_{10}$ cfu/cm^2 versus 7.6 ± 0.50 $\log_{10}$ cfu/cm^2; $P = 0.001$) and larger biomass (OD 1.1 ± 0.09 versus 0.97 ± 0.13; $P = 0.05$) than those originating from resolved infections.

All antimicrobial regimens led to a significant reduction in cfu, with no significant differences between them (Figure 1). By contrast, no significant reduction in biomass was observed with either regimen. Of note, a remarkable dispersion in the response to antibiotics on cfu counting was observed across all strains evaluated, with interstrain coefficients of variation for levofloxacin, rifampicin and levofloxacin + rifampicin of 22.8%, 35.8% and 34.5%, respectively (see range values, Table S3). Lower MBEC values did not correlate with a greater decrease in cfu/cm^2 (Figure S2).

Dynamic biofilm model (CDC biofilm reactor)

For this model, we chose two staphylococcal strains with a common genotypic background, (CC45, same *agr* genotype) and different outcome (failure in *Fail*401 and success in *Cure*806) (Table S1). PK validation of the model was sound (Table S2). Levofloxacin [Figure 2(a)] was more effective than control against both strains, but higher activity was observed against *Cure*806 at 56 h as compared with *Fail*401: −2.5 ± 0.3 $\log_{10}$ cfu/cm^2 versus −1.4 ± 0.2 $\log_{10}$ cfu/cm^2 ($P = 0.08$). In the case of rifampicin monotherapy [Figure 2(b)], there was initial bacterial killing in both cases, which was significantly more pronounced in the *Fail*401 isolate than the *Cure*806 isolate (at 8 h, −1.6 ± 0.4 $\log_{10}$ cfu/cm^2 versus −0.6 ± 0.5 $\log_{10}$ cfu/cm^2; $P = 0.002$) Both staphylococci showed subsequent regrowth from 24 h onwards, in parallel with the emergence

of resistance, which included 100% of the bacterial population at 48 h (Figure S3). The levofloxacin+rifampicin combination was the most effective therapy at 56 h for the *Fail*401 strain and showed an indifferent effect against *Cure*806 strain, with no major differences between the two isolates [Figure 2(c)]. No resistant strains were detected when using levofloxacin either alone or in combination with rifampicin.

Discussion

In the present study, we observed marked variability in response to antibiotics in a clinical collection of biofilm-embedded *S. aureus*. As expected, levofloxacin and rifampicin, alone and in combination, were active against these staphylococcal biofilms, but not to the same extent. The differences occurred despite the fact that the strains were susceptible to and had similar exposures to both antimicrobials. Our experiments focused on the anti-biofilm activity of antimicrobials by excluding other aspects of infections (e.g. intracellular bacteria, host immunity or host metabolomics), therefore the observed variability relied on the genotypic and phenotypic differences of the strains studied. Additionally, the inclusion of a number of strains with different genetic backgrounds reflects the variability found in the clinical setting.[14–16]

In the static model, we were able to test a number of strains by assessing the density of viable biofilm-embedded bacteria as well as overall biomass. While the differences in antimicrobial activity observed were probably reflections of different realities (viable cells versus biomass), heterogeneity of antimicrobial efficacy was still observed with both methods. In contrast, the CDC biofilm reactor is a more demanding model, in which the number of strains that can be tested is limited. However, again, significant variability in the response to rifampicin and levofloxacin monotherapies was observed. Importantly, these differences were attenuated when the strains were challenged with the two drugs in combination. This might suggest that the microbiological variability intrinsic to a given monotherapy could be neutralized by the addition of a second active antibiotic, thus ensuring reliable and reproducible bacterial killing. Determining antimicrobial susceptibility of biofilms in clinical practice remains not feasible. While MBEC and MBIC values have been proposed as alternative indices,[1] a clear correlation with the outcome of these infections has not been proved.[6] Indeed, our results do not support their utility, stressing the important differences of biofilm formation and maturity in the Calgary Device as compared with the clinical setting.[17]

The variability shown in our experiments underlines the importance of the specific infection-causing strain, together with other important aspects, such as the quality of surgical management, the adequacy of antimicrobial treatment and the host's baseline conditions. In this regard, it was interesting to note that strains from PJI cases with a poor prognosis had greater biofilm-forming ability than those from cases with a good prognosis, supporting the relevance of each bacterial strain's background for the patient's prognosis.

Our study has some limitations. First, the concentrations of antimicrobials were lower than those used elsewhere,[13] which may have affected the overall response. Nevertheless, these concentrations would be expected in bone tissue and are also the same ones we used in a previous intracellular *in vitro* model.[8] Second, the number of isolates included in the resolved group ($n=8$) is unbalanced with respect to the recurrent group ($n=3$), which may potentially lead to unstable statistical analyses. Finally, we did not carry out an analysis of virulence factors, which could account for the observed variability, nor a transcriptomic analysis of the genome of each strain when embedded in biofilm and in response to the antimicrobial stress. This could shed some light on the variability observed and should be addressed in future studies.

To conclude, our results emphasize the significant variability that may be observed in response to antimicrobials in the biofilm setting. This heterogeneity may be the consequence of specific genotypic and phenotypic features of the strain responsible for the infection beyond the standard antimicrobial susceptibility profile, and could potentially be a significant parameter of prognosis of the infection.

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Transparency declarations

We declare that we have no conflicts of interest.

Supplementary data

Supplementary Methods, Figures S1–S3 and Tables S1–S3 are available as Supplementary data at *JAC* Online.

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