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Short Communication

# Intraosteoblastic activity of levofloxacin and rifampin alone and in combination against clinical isolates of meticillin-susceptible *Staphylococcus aureus* causing prosthetic joint infection

María Ángeles Meléndez-Carmona[a], Irene Muñoz-Gallego[a], Esther Viedma[a], Jaime Lora-Tamayo[b,*], Fernando Chaves[a]

[a] Department of Clinical Microbiology, Hospital Universitario 12 de Octubre, Madrid, Spain
[b] Department of Internal Medicine, Hospital Universitario 12 de Octubre, Madrid, Spain

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ABSTRACT

*Background:* *Staphylococcus aureus* may invade and persist intracellularly in prosthetic joint infections (PJIs). Despite optimized treatments with levofloxacin plus rifampin, the intracellular reservoir may lead to infection relapse. This study assessed the intracellular activity of levofloxacin and rifampin in an in-vitro model of human osteoblastic infection.

*Methods:* Ten meticillin-susceptible *S. aureus* strains were used to infect osteoblastic MG63 cells. Osteoblasts were challenged with rifampin and levofloxacin at cortical and cancellous bone concentrations. Efficacy was measured as the intracellular counts of colony-forming units ($\log_{10}$CFU) compared with untreated controls. The emergence of small colony variants (SCVs) was determined, and the results were stratified according to the patient's prognosis (six cured and four with persistence/relapse).

*Results:* All regimes led to a significant decrease in CFU count compared with controls (1–2 $\log_{10}$CFU). Levofloxacin was the most effective treatment at both cortical and cancellous bone concentrations (-2.4 to -1.9 $\log_{10}$CFU, respectively). The addition of rifampin to levofloxacin did not improve performance (-1.9 $\log_{10}$CFU for cortical concentration and -1.8 $\log_{10}$ CFU for cancellous concentration). An increase in SCVs was observed in the presence of rifampin. The efficacy of antimicrobials was higher and the formation of SCVs was lower against strains belonging to PJIs with a favourable outcome.

*Conclusions:* Levofloxacin plus rifampin had good intracellular activity against *S. aureus*. However, from the intracellular perspective, the addition of rifampin to levofloxacin showed no benefit but could account for an increased number of SCVs.



## 1. Introduction

Prosthetic joint infections (PJIs) are considered to be difficult to treat due to the presence of bacterial biofilm. Standard antimicrobial treatments are usually ineffective, mainly due to sub-optimal drug concentrations and the development of tolerance to antibiotics among biofilm-embedded bacteria. In addition, micro-organisms responsible for the infection may invade the host cells, thus evading the immune system. *Staphylococcus aureus*, which stands as a paradigmatic pathogen responsible for PJIs due to its ability to colonize bone matrix and evade the immune system [1,2], may also produce intracellular infection, both in its normal-size form and also as small colony variants (SCVs) [3,4]. These adaptive mechanisms enable the micro-organisms to persist intracellularly for long periods, thus evolving from acute infection to chronic and relapsing disease. In order to overcome these difficulties, treatment for PJIs needs to be aggressive with a combined surgical and medical approach, including prolonged courses of antimicrobials [5]. While current guidelines stress that these antibiotics must have good activity against biofilm-embedded bacteria, an emphasis on their intracellular activity has not always been highlighted [6,7].

Levofloxacin plus rifampin is the treatment of choice for acute staphylococcal PJI managed with debridement, antibiotics and implant retention (DAIR) [7,8]. This regime is bactericidal and highly active against biofilm-embedded staphylococci, has good bioavailability and bone diffusion, and has been shown to prevent the selection of resistant mutants compared with rifampin alone [9]. Some previous studies have reported the intracellular activity of both drugs against *S. aureus*, but only a few studies have focused

* Corresponding author. Address: Department of Internal Medicine, Hospital Universitario 12 de Octubre, Av. Córdoba S/N, Madrid 28041, Spain. Tel.: +34 917792406.
*E-mail address:* sirsilverdelea@yahoo.com (J. Lora-Tamayo).

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**Table 1**
Minimum inhibitory concentrations (MICs) and clinical outcomes.

| Strain number | Clonal complex | Treatment duration (days) | Rifampin MIC (mg/L) | | Levofloxacin MIC (mg/L) | | Outcome[a] |
|---|---|---|---|---|---|---|---|
| | | | pH 7.4 | pH 5.0 | pH 7.4 | pH 5.0 | |
| 117-1 | 1021 | 25 | 0.016 | 0.002 | 0.19 | 0.50 | Success |
| 116-1 | 5 | 44 | 0.012 | 0.003 | 0.19 | 0.50 | Success |
| 804-1 | 10 | 75 | 0.008 | 0.004 | 0.13 | 0.50 | Success |
| 221-1 | 30 | 91 | 0.008 | 0.006 | 0.19 | 0.50 | Success |
| 111-1 | 30 | 68 | 0.008 | 0.002 | 0.25 | 0.75 | Success |
| 806-1 | 45 | 46 | 0.012 | 0.003 | 0.13 | 0.38 | Success |
| 201-1 | 22 | 84 | 0.008 | 0.002 | 0.19 | 0.50 | Failure |
| 401-1 | 45 | 25 | 0.008 | 0.003 | 0.13 | 0.50 | Failure |
| 203-1 | 22 | 125 | 0.008 | 0.003 | 0.19 | 0.50 | Failure |
| 104-1 | 15 | 33 | 0.012 | 0.002 | 0.25 | 0.38 | Failure |

MICs of rifampin and levofloxacin on the tested strains were assessed using the E-test method with Mueller-Hinton agar at pH 7.4 and pH 5.00 and outcome of patients.
[a] Treatment was considered to have failed when the patient needed salvage therapy due to persistent or relapsing staphylococcal infection.

on their efficacy at clinically realistic concentrations in bone, and no studies have examined the activity of a combination of levofloxacin plus rifampin. Therefore, this study aimed to assess the intracellular activity of levofloxacin and rifampin, alone and in combination, against a clinical collection of meticillin-susceptible *S. aureus* (MSSA) strains with different genetic backgrounds, but all responsible for PJI managed with DAIR and treated with levofloxacin plus rifampin. This study also aimed to investigate the effect of these antimicrobials on the development of intracellular SCVs in an in-vitro model of human osteoblastic cell infection.

## 2. Materials and methods

### 2.1. Bacterial strains and susceptibility testing

Ten clinical MSSA PJI clinical isolates belonging to different clonal complexes were selected at random from a prospective multicentre study [10]. All patients had an acute PJI (two haematogenous and eight early post-surgical infections) managed with DAIR (debridement <21 days after onset of symptoms), and their treatment had included rifampin (600 mg once daily) plus levofloxacin (750 mg once daily). A reference strain (MSSA ATCC 25923) was used as the control. Minimum inhibitory concentrations (MICs) were evaluated by E-test using Mueller-Hinton agar following the recommendations of the EUCAST guidelines. In addition, MICs were evaluated at pH 5 by adding hydrochloric acid to mimic the conditions expected within lysosomes. All isolates were susceptible to rifampin and levofloxacin. The antimicrobial susceptibility profiles of the isolates used in this study and the clinical outcomes of patients are summarized in Table 1.

### 2.2. MG63 osteoblastic cell culture and intracellular infection

The intracellular activity assay was performed as described previously on MG63 (CRL-1427) human osteoblastic cell line [6]. Cells were grown in Dubelcco Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum with 100 μg/mL gentamicin. MG63 osteoblasts were seeded at 40,000 cells per well into 24-well tissue culture plates, and incubated until 70–80% confluence. Prior to assay, confluent cells were washed with phosphate-buffered saline (PBS) and incubated with DMEM without gentamicin. Next, cells were infected with bacterial suspensions in the stationary phase at a multiplicity of infection of 100. After 2 h of infection, the cell culture was washed twice with PBS and incubated for 1 h with DMEM supplemented with 10 μg/mL lysostaphin to eliminate the extracellular and non-internalized bacteria [6]. All supernatants were plated on agar blood plates to ensure that all bacteria were intracellular.

### 2.3. Antimicrobial intracellular activity

Antimicrobial regimes were added to infected cells and incubated for 24 h in DMEM. Lysostaphin was added to the media to prevent *S. aureus* released from cells from re-infecting new cells. After 24 h of incubation at 37°C, osteoblasts were washed three times with PBS and lysed with sterile water. Cell lysates were plated on agar blood plates for quantitative counts. After overnight incubation, the wild-type colonies were counted. The definition of SCVs was based on the appearance of pinpoint colonies or colonies with a size less than ~1/10 of the wild-type strain measured at 48 h.

Antimicrobial regimes included rifampin, levofloxacin and their combination. These antimicrobials were tested in a range of concentrations that could be expected in cortical and cancellous bone of humans with standard dosages of levofloxacin (750 mg) and rifampin (600 mg) [11–16]: in cortical bone, the estimated concentrations for rifampin and levofloxacin were 2.5 mg/L and 3 mg/L, respectively; and in cancellous bone, the estimated concentrations were 5 mg/L and 6 mg/L, respectively (Table S1, see online supplementary material). Each antimicrobial agent and the combination were evaluated against each staphylococcal strain in three independent experiments.

### 2.4. Clinical outcomes of patients

Patients were treated with levofloxacin plus rifampin for a median of 57 days after debridement [interquartile range (IQR) 29–88]. Six patients were considered to be cured after a median follow-up of 1.23 years (range 1.16–1.76) and treatment was considered to have failed in four patients (Table 1). Treatment was considered to have failed when the patient needed salvage therapy due to persistent or relapsing staphylococcal infection. No significant differences regarding the length of treatment, the use of rifampin or the administration of levofloxacin plus rifampin were observed between patients with good and bad outcomes.

### 2.5. Statistical analysis

Intracellular activity of the antimicrobials was assessed by the decrease in *S. aureus* intracellular load observed for each treatment compared with untreated cells. Bacterial counts were expressed as the log of the number of colony-forming units ($\log_{10}$CFU) per 100,000 osteoblasts. The median and IQR of the results observed

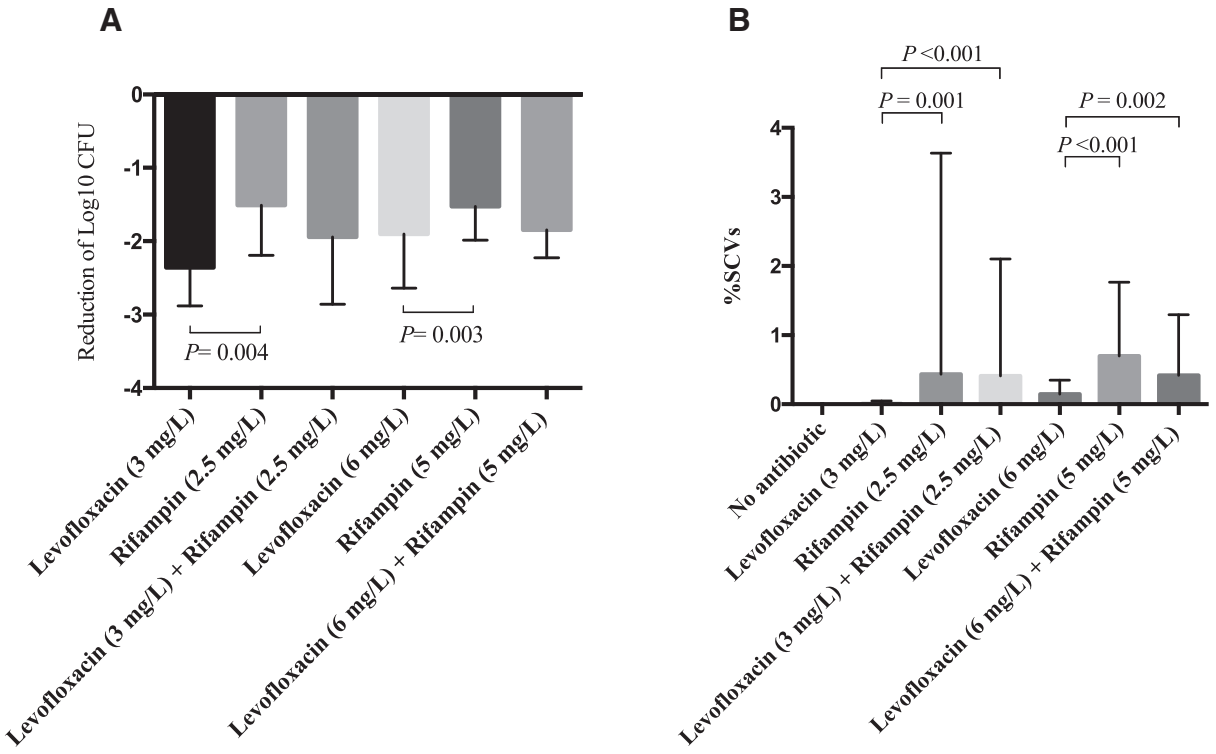


**Fig. 1.** Intracellular effect of antibiotics at cortical and cancellous bone concentrations against meticillin-susceptible *Staphylococcus aureus* isolates causing prosthetic joint infections. (A) Median intracellular count reduction and interquartile range (IQR); all antibiotic regimes, alone and in combination, showed a significant reduction in $\log_{10}$ colony-forming units (CFU) in relation to control (no antibiotic) set to zero ($P<0.001$). (B) Median percentage of small colony variants (SCVs) and IQR; at both cortical and cancellous bone concentrations, rifampin alone and in combination showed a significant increase in the percentage of SCVs in comparison with untreated cells (no antibiotic).

for each treatment regime for the 10 clinical strains were used. Likewise, the amount of intracellular SCVs at the end of the experiment was expressed using the ratio of SCVs in the presence of antimicrobial agent compared with untreated cells. Finally, the intracellular activity of antimicrobials was analysed according to the clinical outcome of the patient. Comparisons were made using the Mann-Whitney *U*-test. All statistical analyses were two-tailed and were made using GraphPad Prism Version 6. $P<0.05$ was considered to indicate statistical significance.

## 3. Results

### 3.1. Antimicrobial intracellular activity

All antibiotic regimes, at both cortical and cancellous concentrations, showed a significant decrease in $\log_{10}$CFU compared with untreated cells ($P<0.01$) (Fig. 1A). Results for cortical bone concentrations were -2.4 [IQR -2.9 to-1.5] $\log_{10}$CFU for levofloxacin 3 mg/L, -1.6 (IQR -2.2 to -0.8) $\log_{10}$CFU for rifampin 2.5 mg/L, and -1.9 (IQR -2.8 to -0.9) $\log_{10}$CFU for the combination of levofloxacin 3 mg/L plus rifampin 2.5 mg/L. Levofloxacin 3 mg/L was more active than rifampin 2.5 mg/L ($P=0.004$). The combination of levofloxacin 3 mg/L plus rifampin 2.5 mg/L was not significantly better than either monotherapy. Results for cancellous bone concentrations were -1.9 (IQR -2.6 to -1.6) $\log_{10}$CFU for levofloxacin 6 mg/L, -1.5 (IQR -2.0 to -1.0) $\log_{10}$CFU for rifampin 5 mg/L, and -1.8 (IQR -2.2 to -1.2) $\log_{10}$CFU for the combination of levofloxacin 6 mg/L plus rifampin 5 mg/L. Levofloxacin 6 mg/L was more active than rifampin 5 mg/L ($P=0.003$). The combination of levofloxacin 6 mg/L plus rifampin 5 mg/L did not show higher activity than levofloxacin as monotherapy ($P=0.201$), but it did show a trend towards higher activity than rifampin 5 mg/L ($P=0.06$). No significant differences were observed in the intracellular activity of each antimicrobial regimen at the two tested concentrations of cortical and cancellous bone.

### 3.2. Emergence of SCVs

SCVs in untreated cells after 24 h of incubation in all PJI isolates were 0% (IQR 0). A significant increase was observed at both rifampin concentrations: 0.4% (IQR 0.02–3.6) and 0.7% (IQR 0.3–1.7) ($P<0.001$); and for the combination of rifampin plus levofloxacin at cortical and cancellous bone concentrations: 0.4% (IQR 0.02–2.1) and 0.4% (IQR 0.2–1.3), respectively ($P<0.001$).

### 3.3. Intracellular activity according to the prognosis

Overall, all regimes showed better intracellular activity against strains recovered from patients with a favourable outcome compared with those isolated from patients in whom treatment failed (Fig. 2A). At cortical concentrations, the combination therapy of levofloxacin plus rifampin showed higher intracellular activity: -2.5 (IQR -3.2 to-1.8) $\log_{10}$CFU vs -1.0 (IQR -1.6 to-0.7) $\log_{10}$CFU ($P=0.002$). Differences were not significant at cancellous concentrations: -2.0 (IQR: -2.2 to -1.5) $\log_{10}$CFU vs -1.7 (IQR -2.2 to -1.0) $\log_{10}$CFU ($P=0.185$).

Likewise, in the absence of antimicrobials, the formation of SCVs was more frequent among patients with an unfavourable outcome than those considered to be cured: 0% (IQR 0–1.6) vs 0% (IQR 0) ($P=0.166$) (Fig. 2B). In the presence of antimicrobials, this difference was more pronounced among strains treated with rifampin 2.5 mg/L [3.2% (IQR 0.8–6.0) vs 0.2% (IQR 0.01–0.6); $P=0.038$] and levofloxacin 3 mg/L plus rifampin 2.5 mg/L [2.1% (IQR 0.7–2.9) vs 0.05% (IQR 0.01–0.6); $P=0.015$].

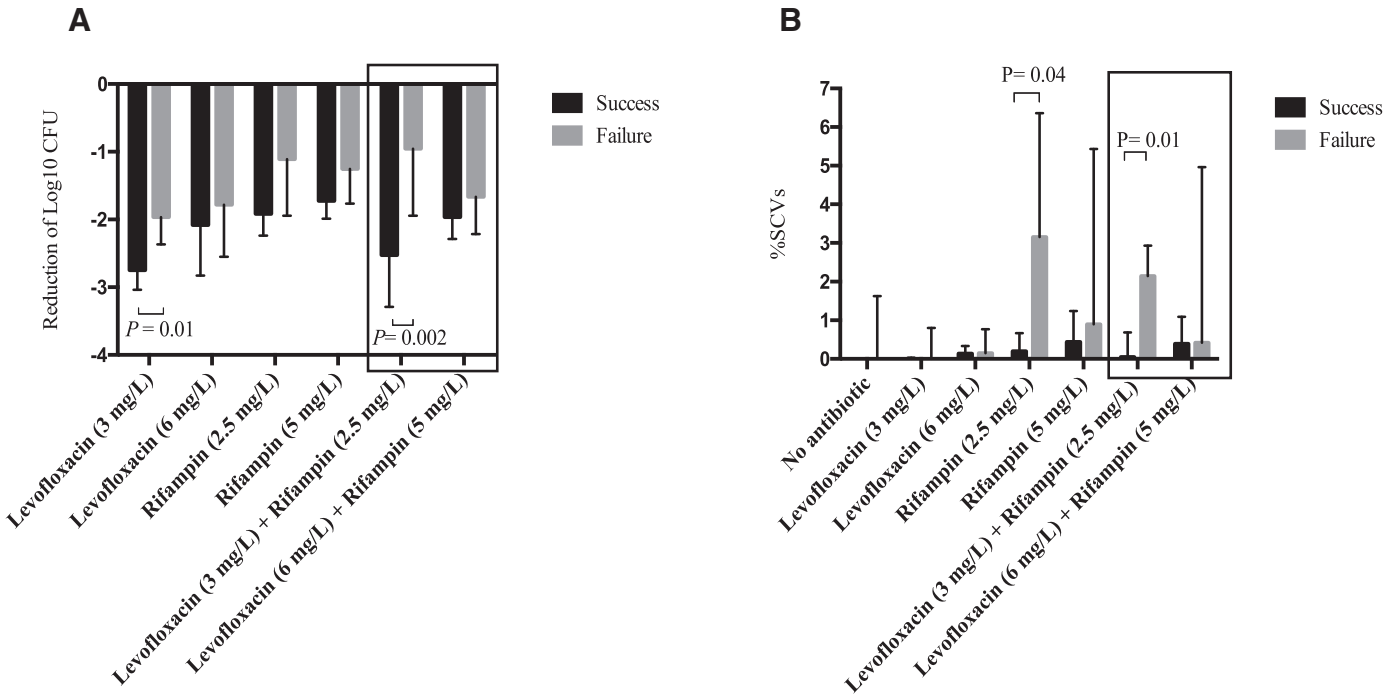


**Fig. 2.** Intracellular activity and emergence of small colony variants (SCVs) in meticillin-susceptible *Staphylococcus aureus* causing prosthetic joint infection, according to clinical outcome. (A) Median intracellular count reduction and interquartile range (IQR) (bars). (B) Median percentage of SCVs and IQR. Treatment was considered to have failed when the patient needed salvage therapy due to persistent or relapsing staphylococcal infection. The squares highlight the actual treatment that the patients received after surgical debridement. CFU, colony-forming units.

## 4. Discussion

This study analysed the intracellular effect of levofloxacin and rifampin in a clinical collection of MSSA responsible for PJIs. The results show that these antibiotics, alone and in combination, significantly reduced the number of intracellular CFU in comparison with untreated osteoblast cells. The magnitude of the effect (≈1–2 $\log_{10}$CFU) is consistent with previous studies assessing the intracellular activity of both antimicrobials at similar concentrations as used in this experiment [11,17,18].

Based on observations from animal experiment models and clinical experience, current guidelines recommend the use of levofloxacin plus rifampin as the treatment of choice for acute staphylococcal PJIs managed with DAIR [8]. Although the role of intracellular staphylococci in the pathogenesis of infection has not always been taken into account, previous studies have highlighted the importance of intracellular bacteria and persistent forms such as SCVs in PJIs and foreign-body-associated infections [3,4]. Therefore, it could be argued that the intracellular activity of antimicrobials could be of importance and add supplementary efficacy in these difficult-to-treat infections. In this regard, previous studies have shown that rifampin and third- and fourth-generation fluoroquinolones are among the most active intracellular antimicrobials against staphylococci [6,11]. These results, focusing on concentrations to be expected in bone tissue in the clinical setting, confirm the good activity of both rifampin and levofloxacin. In contrast to Barcia-Macay et al. [11], a dose-related effect was not observed in the present study, but this could be explained by the reduced range of concentrations used in this study.

To the best of the authors' knowledge, this is the first study to assess the intracellular effect of the combination of levofloxacin plus rifampin. Overall, levofloxacin showed the highest activity, and the addition of rifampin was mostly indifferent or showed a non-significant decrease in the activity of levofloxacin. However, in some cases, it led to a significant increase in the number of SCVs. It remains uncertain whether the magnitude of this increase (≈1–2%) is clinically significant, but it is believed that this observation could be of importance as chronic and recurrent infections are associated with the presence of the SCV phenotype, and its development can be induced by the intracellular medium or by selective antibiotic pressure [19].

This study found an indifferent effect of the combination of levofloxacin plus rifampin compared with monotherapies. The antagonistic effect of rifampin on the activity of other drugs against planktonic bacteria was described many years ago [20], and it has also been shown against biofilm-embedded bacteria in an experimental animal model [21]. Although the development of resistance for rifampin cannot be excluded, the present data in the cellular model could partially support the results observed in these studies, and would also suggest that monotherapy with last-generation fluoroquinolones (i.e. levofloxacin, moxifloxacin), with higher intrinsic antistaphylococcal activity compared with ciprofloxacin, could have a potential role in the setting of PJI. In this regard, the recently released fluorquinolone, delafloxacin, with a weak acid character, showed enhanced activity in acidic environments, which could be an advantage over other fluoroquinolones in the treatment of these PJIs [22]. However, caution is required as the model used in this study only regarded the intracellular activity of antimicrobials, with no reference to other relevant aspects of PJI, such as the role of biofilm-embedded bacteria, planktonic bacteria released from biofilm, or the inflammatory response to infection.

Stratification of the results according to clinical outcome shows higher intracellular activity on strains responsible for infection with a favourable evolution, compared with strains associated with treatment failure. From an overall perspective, these patients had similar surgical and antimicrobial treatment, and, in all cases, the infection was caused by staphylococci susceptible to both levofloxacin and rifampin. Bearing this in mind, the study observations highlight the importance of the bacteria's genotypic background and phenotypic behaviour beyond the species and the

antibiogram, and therefore the need for deepening out understanding of the intimate genomic and proteomic pathways of microorganisms.

Some limitations of this study must be taken into account. First, actual intracellular concentrations of antimicrobials were not assessed. However, this is predictable [11], and the study aimed to examine intracellular activity of antimicrobials at reproducible extracellular concentrations. Second, intracellular activity of tested antibiotics was only measured at 24 h. In this regard, Barcia-Macay et al. reported that an important aspect in the intracellular activity of rifampin is its pronounced activity at 3–6 h, which not only does not progress afterwards, but may decrease [11]. Thus, it would be interesting to study the intracellular role of the combination for longer periods and at time points other than 24 h. Third, the difficulties in identifying SCVs and their clinical meaning must be interpreted with caution. In this regard, the differences in the emergence of SCVs may reflect particular molecular pathways in some staphylococcal strains, which may help explain the pathogenesis and prognosis of these infections; however, the clinical implications remain unknown. Finally, the study observations were based on the aggregated efficacy of the entire strain collection, without focusing on any of them. While the results may be variable for each specific staphylococci, the use of 10 strains with different phenotypic and genotypic characteristics may provide an overall idea of the performance of the antibiotics studied, and increase the external validity of the results.

To conclude, both levofloxacin and rifampin show significant intracellular activity against *S. aureus*. From the intracellular perspective, the addition of rifampin to levofloxacin did not ameliorate its activity, but could increase the emergence of SCVs. Further studies are necessary to explore the role of this combination against the intracellular component of PJIs which may lead to a better therapeutic approach for patients.

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## Competing interests

None declared.

## Ethical approval

This study was approved by the Research Ethics Committee of the Health Research Institute, Hospital Universitario 12 de Octubre, Madrid, Spain (Ref. TP16/0092).

## Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.06.018.

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