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Research Paper

Pharmacodynamic assessment of vancomycin–rifampicin combination against methicillin resistant *Staphylococcus aureus* biofilm: a parametric response surface analysis

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Abstract

Objectives A combination of vancomycin and rifampicin (rifampin) is commonly used to treat staphylococcal infections but its efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm is controversial. The objective of this study was to use a recently developed quantitative methodology to characterise the killing effect of vancomycin and rifampin combination against MRSA biofilm.

Methods MRSA biofilm was exposed to escalating concentrations of vancomycin and rifampin and the viability of the biofilm-ensconced bacteria was evaluated. ADAPT II was used to model the concentration–effect relationship and determine the optimal sampling concentrations. Combination experiments were then conducted and the observations were compared with a simulated response surface representing null interaction. Finally, the pharmacodynamic interaction index (PDI) was computed as the ratio of the volumes under the observed and simulated surfaces.

Key findings In the combination experiments, all observations showed an inferior antibacterial effect to what is expected under null interaction assumption and the PDI was estimated to be 3.36 (95% CI, 3.25 to 3.46).

Conclusions The results of the study demonstrate in-vitro antagonism between vancomycin and rifampin against MRSA biofilm. The quantitative approach employed to quantify the antibacterial effect of the combination provides a scientific rationale for further in-vivo investigations that should allow a better understanding of the therapeutic potential of this combination in biofilm-associated MRSA infections.

Keywords biofilm; MRSA; response surface; rifampin; vancomycin

Introduction

Staphylococcus aureus is one of the most common Gram-positive pathogens encountered in both community and hospital settings.[1] Methicillin-resistant *S. aureus* (MRSA) accounts for 40% of all nosocomial *S. aureus* infections[2] and 64% of *S. aureus* infections in intensive care units.[3] MRSA infections are associated with higher morbidity, mortality and healthcare cost than methicillin-sensitive *S. aureus* (MSSA) infections[4,5] with some studies reporting the mortality rate from MRSA bacteraemia to be higher than 50%.[6,7]

Although vancomycin has been the standard therapy for MRSA infections, staphylococcal isolates with decreased susceptibility to vancomycin, known as vancomycin-intermediate *S. aureus* (VISA), have been reported worldwide.[8–10] Moreover, the polymorphism that is responsible for this decreased susceptibility was also found to be associated with overproduction of biofilm.[11] Biofilm is a microbial derived sessile community characterised by cells that are reversibly attached to a substratum or interface or to each other, are embedded in a matrix of polymeric substances that they have produced and exhibit an altered phenotype with respect to growth rate, antimicrobial resistance and gene transcription.[12] *S. aureus* is known to colonise and form biofilm on indwelling medical devices and intravascular catheters resulting in device-related and catheter-related bloodstream infections.[12,13] Biofilm-associated infections tend to be persistent and very difficult to eradicate because of the inherent resistance of biofilm-embedded bacteria.[14]

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Due to the current decline in development of novel antimicrobial agents,[15] the use of combination therapy has gained attention as an alternative strategy for combating biofilm resistance. Rifampicin (rifampin) is a bactericidal agent that is active against *S. aureus* but is often used in combination to avoid the rapid emergence of resistance.[16,17] This agent has been reported to have a strong anti-biofilm activity that could be attributed to its ability to penetrate the biofilm[16] or its ability to inhibit the adherence of the bacteria to surfaces.[18] Nevertheless, the efficacy of its combination with vancomycin against MRSA biofilm remains controversial,[16–24] despite the common use of this combination for treatment of staphylococcal infections.[25,26] A response surface analysis approach that involves pharmacodynamic modelling and simulation has recently been proposed for quantitative evaluation of antimicrobial agents interactions.[27] The objective of this study was to use the new methodology to characterise the bactericidal effect of vancomycin and rifampicin separately and in combination against MRSA biofilm.

Materials and Methods

Bacterial strain and antimicrobial agents

Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (American Type Culture Collection, Manassas, USA) was used in the study. Before each experiment, MRSA ATCC 43300 was sub-cultured twice on Tryptic Soy Broth (TSB) and incubated for 16 h at 37°C. The inoculum was then prepared in cation-adjusted Mueller Hinton II broth (MHII) and diluted to match 0.5 McFarland standard, which is equivalent to 1.5×10^8 CFU/ml.

Vancomycin and rifampicin powders were purchased from Sigma-Aldrich (St Louis, USA). Solutions of 20 mg/ml vancomycin and 6.4 mg/ml rifampicin were prepared and stored as stock solutions at −80°C according to the Clinical & Laboratory Standard Institute (CLSI) guidelines.[28] Samples of the stock solutions were thawed at room temperature and diluted in MHII broth before experiments.

Planktonic susceptibility testing

The minimum inhibitory concentrations (MICs) of the antimicrobial agents were determined using the broth microdilution method as described by CLSI guidelines.[28] The experiments were performed in polystyrene, round-bottom, 96-wells microplates (Greiner, Monroe, USA). Twofold serial dilutions of the antibiotics were used and the final bacterial count in each well was 5×10^5 CFU/ml. The MIC was defined as the lowest concentration of the antibiotic that resulted in no visible growth after aerobic incubation at 37°C for 24 h.

Biofilm susceptibility testing

Biofilm formation

Seventy-five-microlitre inoculums of 1.5×10^8 CFU/ml TSB culture were incubated for 24 h at 37°C in polystyrene, round-bottom, 96-wells microplates.[29] After incubation, the supernatant was aspirated and the wells were washed twice with sterile normal saline solution.

Minimum biofilm inhibitory concentration (MBIC)

One-hundred microlitres of two-fold serial dilutions of the antibiotics in MHII were added to the wells with the established biofilms. After incubation for 18 h at 37°C, the plates were examined visually for bacterial growth indicated by the presence of turbidity. The MBIC was defined as the lowest concentration of the antibiotic that resulted in no visible growth.[30]

Minimum biofilm bactericidal concentration (MBBC)

Ten-microlitre volumes from wells with no visible growth were transferred into a new 96-well plate and diluted with 90 μl of TSB to minimise the carryover effect. After incubation for 24 h at 37°C, the plates were examined visually for bacterial growth. The MBBC was defined as the lowest concentration of the antibiotic that prevented visible growth.

Biofilm time-kill studies

Biofilm formation

MHII broth (0.5 ml containing 1.5×10^6 CFU/ml of the microorganism) was used to inoculate 1.5-ml polypropylene tubes (Greiner, Monroe, USA).[31] The tubes were incubated for 24 h at 37°C under aerobic condition without shaking. The supernatant was then carefully aspirated and the tubes were washed with normal saline solution. Establishment of MRSA biofilm in the tubes was confirmed using scanning electron microscopy according to the method described by van Heerden *et al*.[32]

Anti-biofilm assessment of single agents

MRSA biofilm was exposed to vancomycin or rifampicin at increasing concentrations of 0 (control), 0.25, 1, 4, 16 and 64 times MBIC. All experiments were run in duplicate. After 24 h at 37°C, the tubes were sonicated for 5 min in an ultrasonic water bath followed by vigorous vortexing for 60 s to dislodge and disperse the cells from the biofilm.[33] After sonication, samples of 100 μl were withdrawn and were ten-fold serially diluted in sterile normal saline solution to minimise the antibiotic carryover effect by reducing the antibiotic concentration to sub-MIC levels. Samples (50 μl) were then plated onto Muller Hinton Agar (MHA) plates to quantify the total biofilm-embedded bacterial burden. After incubation of the MHA plates at 37°C for 24 h, the viable cell count was determined for different treatments and controls.

Pharmacodynamic modelling

The total bacterial burdens after 24 h of antibiotic exposure were logarithmically transformed and fitted to an inhibitory sigmoid Emax model in ADAPT II (Biomedical Simulation Resource, University of Southern California, Los Angeles, USA) using the maximum-likelihood estimation method.[34] The observations were weighted by the reciprocal of their variances. The baseline effect was fixed to the logarithm of the mean bacterial count observed after 24 h in the control experiments.

Determination of optimal sampling concentrations

The parameters estimates obtained from the sigmoid Emax model were assumed to be the true parameter values and were used in ADAPT II to determine four optimal and clinically achievable sampling concentrations that would most precisely estimate the model parameters for each antibiotic. D-optimality criterion was employed to minimise the determinant of the variance–covariance matrix of the estimated parameters, or equivalently, to minimise the volume of the confidence region for the parameter estimates. The upper bounds of the concentration constraints were the maximum clinically achievable concentrations of the two antibiotics (64 μg/ml for vancomycin and 32 μg/ml for rifampicin). A conservative lower bound of 0.25 × MBIC was used to characterise the whole pharmacodynamic profile and identify any synergistic interactions at low concentrations.

Anti-biofilm assessment of the combination

MRSA biofilm was established in the same way described above for the single agent experiments. Twenty-five combinations of the optimal sampling concentrations (including control) of the two agents were then assessed for their bactericidal activity against MRSA biofilm. After 24 h of exposure, the total bacterial burden was retrieved, quantified and used to construct a three-dimensional response surface. Using effect summation, another three-dimensional response surface was simulated to describe the predicted combined antibacterial effect in case of null interaction as follows[27]:

$$Effect_{combination} = E_{vancomycin} + E_{rifampin} \quad (1)$$

$$LogCFU/ml = E_o - \left\{\left[\frac{E_{\max r}.C_r^{Hr}}{C_r^{Hr} + C_{50r}^{Hr}}\right] + \left[\frac{E_{\max v}.C_v^{Hv}}{C_v^{Hv} + C_{50v}^{Hr}}\right]\right\} \quad (2)$$

Where E_o represents the mean bacterial burden in the control experiments, E_{maxr} and E_{maxv} are the maximum effects of rifampicin and vancomycin, C_r and C_v are the concentrations of rifampicin and vancomycin, C_{50r} and C_{50v} are the concentrations of rifampicin and vancomycin at 50% of the maximum effect, H_r and H_v are the Hill factors for rifampicin and vancomycin, respectively.

Computation of the pharmacodynamic interaction index

The volume under the simulated surface was estimated by double integration of Equation 2 over the clinically achievable range. The volume under the observed data was estimated by linear interpolation between the observed data then estimating the volumes of the cuboids formed. These volumes can be conceptualised as the integral bactericidal effect over the studied concentration ranges of the two antibiotics.[27] A 95% confidence interval (CI) of the volume under the observed surface was calculated using the confidence intervals of the mean observed data (mean ± 1.96 × standard deviation (SD)). The pharmacodynamic interaction index was computed as the ratio of the volumes under the observed and simulated surfaces. Synergy and antagonism were defined as interaction index values of <1.0 and >1.0, respectively. Matlab (version 7.1, The MathWorks, Natick, USA) was used for computation of the volumes and visualisation of the results. The Matlab code as well as the Fortran code used in ADAPT II for pharmacodynamic modelling and generation of the D-optimal concentrations are available from the authors.

Results

The results of the susceptibility experiments are shown in Table 1. The MIC and MBIC values are consistent with those presented in previous reports.[20,35] Although the MBIC of both agents were comparable, the MBIC was more than 800-fold the MIC in the case of rifampicin while it was only 8-fold higher for vancomycin.

The methodology adopted for biofilm formation and quantification in the time-kill studies was highly reproducible with less than 3.5% variability in the control experiments results across the study period. The mean bacterial density retrieved from the biofilm in the control experiments was 3.2×10^9 CFU/ml. Rifampicin exhibited a superior antibacterial profile to vancomycin against MRSA biofilm (Figure 1). In the pharmacodynamic modelling, the sigmoid inhibitory Emax model fitted the data adequately, with R^2 of 0.97 and 0.99 for vancomycin and rifampicin data, respectively (Figure 1). Table 2 shows the parameter estimates for both antibiotics as well as the precision associated with their estimation. The uncertainty in the parameter estimates was generally low, with the highest relative standard error% (RSE%) being 28.6%. The sampling concentrations of the D-optimal design were estimated to be 2, 4.1, 12.8 and 64 μg/ml for vancomycin and 1.56, 4.2, 14.4 and 32 μg/ml for rifampicin.

Figure 2 shows the parametric response surface that presents the additive anti-biofilm activity of the combination simulated under null interaction assumption and calculated using Equation 3.

$$LogCFU/ml = 9.5 - \left\{\left[\frac{7.27xC_r^{0.86}}{C_r^{0.86} + 5.72^{0.86}}\right] + \left[\frac{3.21xC_v^{1.34}}{C_v^{1.34} + 3.56^{1.34}}\right]\right\} \quad (3)$$

Table 1 Susceptibility of MRSA 43300 in the planktonic and biofilm states to vancomycin and rifampicin

Antimicrobial agent	MIC (μg/ml)	MBIC (μg/ml)	MBBC (μg/ml)
Vancomycin	1	8	32
Rifampicin	0.0075	6.25	6.25

MIC, minimum inhibitory concentration in the planktonic state; MBIC, minimum biofilm inhibitory concentration; MBBC, minimum biofilm bactericidal concentration.

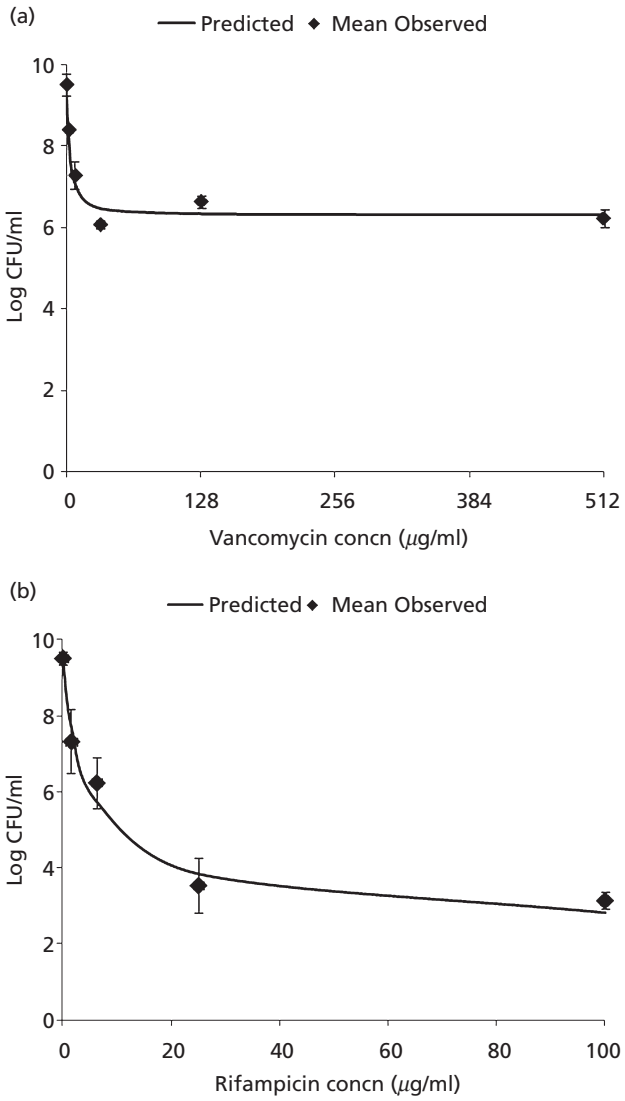

Figure 1 Model fit of the total bacterial density after exposure of biofilm to varying concentrations of vancomycin (a) or rifampicin (b) for 24 h in the single agent experiments. Data are shown as means ± SD.

Table 2 Parameter estimates of the pharmacodynamic models of vancomycin and rifampicin

Parameter	Estimate (%RSE)	
	Vancomycin	Rifampicin
E_{max}	3.21 (3.4)	7.27 (4.9)
EC_{50}	3.56 (16.9)	5.72 (28.6)
H	1.34 (21.7)	0.86 (27.1)

The bacterial densities observed at the different combination concentrations are demonstrated in Figure 3. Observations showed lower anti-biofilm activity than the simulated profile at all concentration combinations (Figure 4). The higher the concentrations of the agents, the higher the antagonism observed, with the highest antagonism observed with the combination of 64 μg/ml of vancomycin and 32 μg/ml of rifampicin (Figure 4).

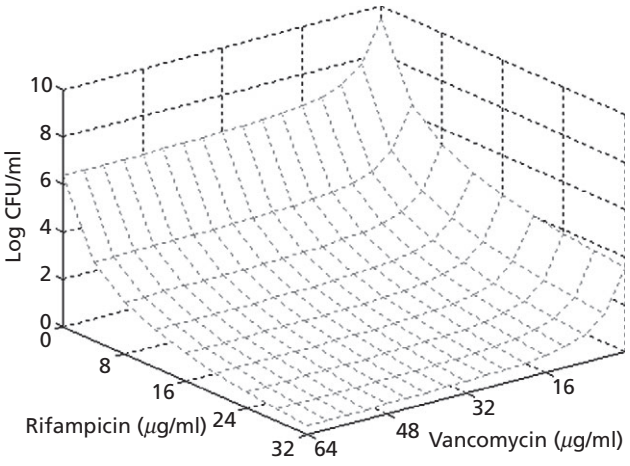

Figure 2 Simulated response surface showing the expected anti-biofilm effect of the vancomycin–rifampicin combination if there is no interaction between the two agents.

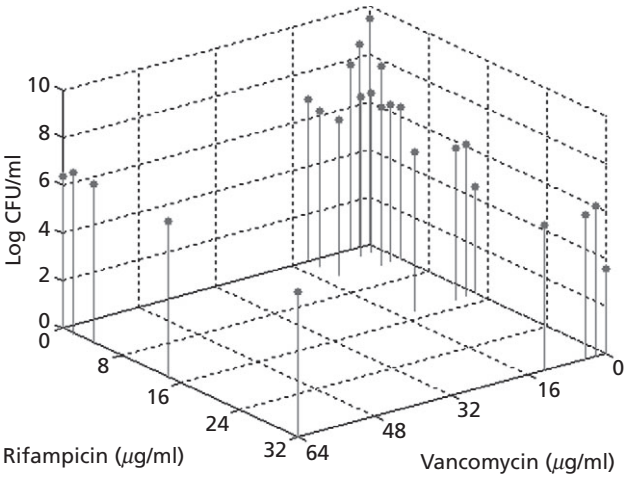

Figure 3 The observed bacterial density after 24 h of biofilm exposure to different concentrations of vancomycin– rifampin combination.

The volume under the simulated surface was 4113.3, while the volume under the observed points was found to be 13 802.1 (95% CI, 13 380.3 to 14 223.9). The pharmacodynamic interaction index was estimated to be 3.36 (95% CI, 3.25 to 3.46).

Discussion

The use of combinations of antimicrobial agents has emerged as a promising therapeutic approach to overcome the increased bacterial resistance and the poor pipeline of novel antimicrobial agents.[36] Since not all antimicrobial combinations act synergistically, use of combination therapy is not always advantageous and approaches that can predict the pharmacodynamic interaction between the combined antimicrobial agents would help make a rational choice of the antimicrobial combinations. Although several in-vitro methods have been used to evaluate antimicrobial combinations, their

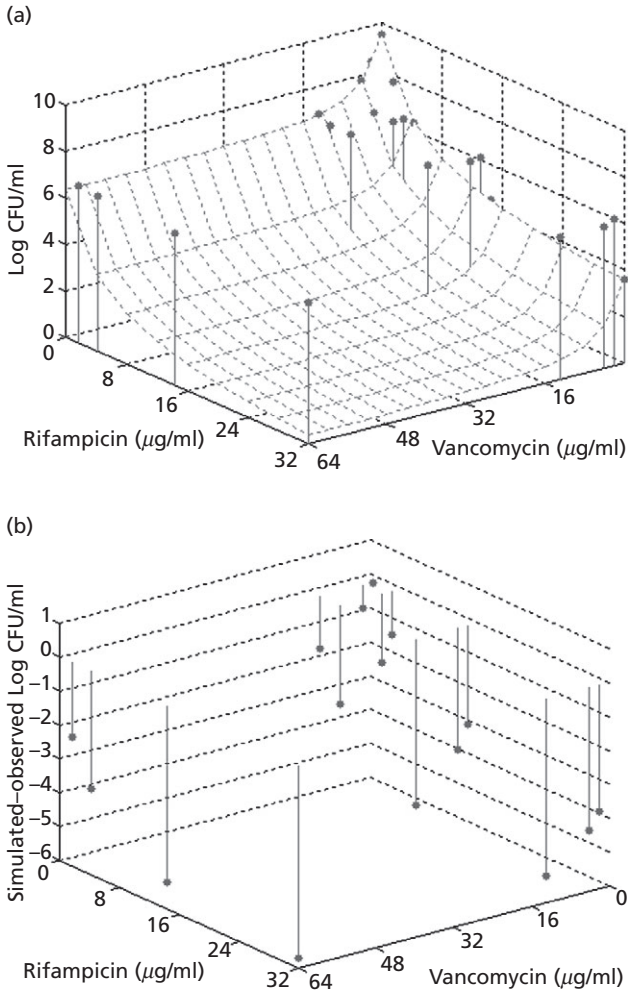

Figure 4 (a) Comparison of the observed (circles) and simulated (mesh) anti-biofilm activity of vancomycin–rifampicin combination at different concentrations. Observations show higher count (i.e. lower effect) than what is expected under null interaction assumption. (b) The extent of antagonism at the different concentrations of the vancomycin–rifampicin combination.

results may not correlate with each other[37–39] and they were often of little value in predicting the clinical outcome as assessed by in-vitro pharmacokinetic models as well as animal and clinical studies.[40–43] In addition, the assumptions that some of these methods are built on have been questioned, which invalidates the interpretation of their results.[44]

Time-kill studies have been commonly used to evaluate antimicrobial combinations. An advantage of this technique is that it allows quantitative assessment of the extent of the bacterial killing effect rather than the dichotomous visual evaluation of bacterial inhibition used in the checkerboard technique.[26] These studies, however, evaluate the antimicrobial interaction at one static concentration and hence results cannot be extrapolated to other concentrations. This limits the clinical relevance of the results given the fact that the drug concentration varies *in vivo* according to its pharmacokinetic parameters. Moreover, there is no widely accepted definition of synergy in time-kill experiments for bactericidal agents.[40]

Tam *et al*. have recently proposed a response surface analysis approach for pharmacodynamic assessment of antimicrobial agents interactions.[27,40] This technique involves conducting time-kill studies at different concentration combinations of the antimicrobial agents and using pharmacodynamic modelling and effect summation to define the parametric response surface representing the additive effect of the combination. The presence of data above or below this response surface indicates antagonism or synergism, respectively. In addition, a pharmacodynamic interaction index is computed to allow a quantitative measure of the interaction. A confidence interval for this index can be estimated as well by including the replicates variability in the analysis to provide a statistical basis for interpreting the results and comparing the different combinations.[40]

In this study, we used the response surface analysis approach to evaluate the efficacy of the vancomycin and rifampicin combination against MRSA biofilm. The effect of the biofilm on the susceptibility to vancomycin and rifampicin was enormous, as demonstrated in Table 1. This is consistent with previous reports relating to the association between biofilm formation and antimicrobial resistance.[12,45] Rifampicin demonstrated higher efficacy than vancomycin against MRSA biofilm in the single-agent time-kill studies. This could be attributed to rifampicin's lower molecular weight and its lesser structure complexity, which enables higher penetration ability through the biofilm matrix compared with vancomycin. Combination experiments revealed antagonism at all concentrations and the interaction index was appreciably higher than 1 suggesting strong antagonism between the two agents against MRSA biofilm.

Studies on the efficacy of vancomycin–rifampicin combination against MRSA biofilm have had conflicting results.[16–24] Rose *et al*.[20] showed that rifampicin has a minimal effect against low and high biofilm-producing MRSA strains while its combination with vancomycin in time-kill experiments was bactericidal against all the strains. Using multiple combination bactericidal testing, Saginur *et al*.[18] reported that vancomycin and rifampicin combination was effective against MSSA biofilm but fusidic acid had to be added to this combination to produce a similar effect against MRSA biofilm. LaPlante and Woodmansee[19] showed that rifampicin did not enhance the activity of vancomycin against MRSA biofilm while it antagonised and delayed the bactericidal effect of another glycopeptide, daptomycin, when tested in time-kill studies. In addition, antagonism between vancomycin and rifampicin has been reported against MRSA in the planktonic state.[46–48] Antagonism between vancomycin and rifampicin could be attributed to the delaying effect of RNA synthesis inhibition on the activity of cell-wall active antibiotics.[19] The heterogeneity in the testing methods used in the previous studies was suggested as the cause of the inconsistency in the results.[16] We believe, however, that the approach employed in our study provides a more robust quantitative assessment of the antimicrobial agents' interactions, which in turn potentiates the clinical relevance of the obtained results. In fact, despite the common use of this combination clinically, clinical studies have failed to show a therapeutic advantage of concomitant administration of rifampicin and vancomycin in the

treatment of MRSA endocarditis with a slight trend in favour of vancomycin monotherapy.[16,22,24,49]

A limitation of our study is the use of one MRSA strain, which limits the generalizability of the results. Therefore, future studies will be directed towards testing the effect of the combination against MRSA clinical isolates. To the best of our knowledge, this is the first time that the response surface modelling approach has been applied to assessing the anti-biofilm effect of an antimicrobial combination. As reported previously,[27] this approach performs better when used for assessing antimicrobial activity against inherently resistant bacteria, which makes its use in biofilm studies one of its best applications. However, this approach may not be convenient for routine clinical laboratory use due to its laborious nature and its use may be limited to research purposes.

Conclusions

In summary, using a new modelling based approach, we have demonstrated an in-vitro antagonism between vancomycin and rifampicin against MRSA biofilm at clinically achievable concentrations. The parametric approach employed to quantify the activity of the combination provides a scientific rationale for further in-vitro and in-vivo investigations, which will allow a better understanding of the therapeutic potential of this combination in biofilm-associated MRSA infections.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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