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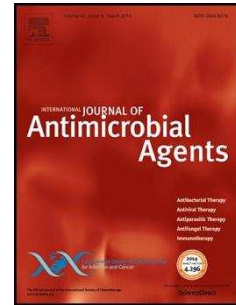
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Efficacy of rifampicin combination therapy for the treatment of enterococcal infections assessed in vivo using a *Galleria mellonella* infection model

Kirsty Skinner ^a, Jonathan A.T. Sandoe ^b, Ranjith Rajendran ^c, Gordon Ramage ^c,
Sue Lang ^{a,*}

^a *Department of Life Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow G4 0BA, UK*

^b *Department of Microbiology, The General Infirmary at Leeds, Leeds LS1 3EX, UK*

^c *Oral Sciences Research Group, Glasgow Dental School, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK*

* Corresponding author. Tel.: +44 141 331 8092; fax: +44 141 311 3208.

E-mail address: sue.lang@gcu.ac.uk (S. Lang).

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Highlights

- Treatment with antibiotic combinations offers the possibility of synergistic killing.
- In vitro observed synergy was validated in vivo using a *Galleria mellonella* model.
- Rifampicin-based combinations proved useful against selected resistant enterococci.

ABSTRACT

Enterococci are a leading cause of healthcare-associated infection worldwide and display increasing levels of resistance to many of the commonly used antimicrobials, making treatment of their infections challenging. Combinations of antibiotics are occasionally employed to treat serious infections, allowing for the possibility of synergistic killing. The aim of this study was to evaluate the effects of different antibacterial combinations against enterococcal isolates using an in vitro approach and an in vivo *Galleria mellonella* infection model. Five *Enterococcus faecalis* and three *Enterococcus faecium* strains were screened by paired combinations of rifampicin, tigecycline, linezolid or vancomycin using the chequerboard dilution method. Antibacterial combinations that displayed synergy were selected for in vivo testing using a *G. mellonella* larvae infection model. Rifampicin was an effective antibacterial enhancer when used in combination with tigecycline or vancomycin, with minimum inhibitory concentrations (MICs) of each individual antibiotic being reduced by between two and four doubling dilutions, generating fractional inhibitory concentration index (FICI) values between 0.31 and 0.5. Synergy observed with the chequerboard screening assays was subsequently observed in vivo using the *G. mellonella* model, with combination treatment demonstrating superior protection of

larvae post-infection in comparison with antibiotic monotherapy. In particular, rifampicin in combination with tigecycline or vancomycin significantly enhanced larvae survival. Addition of rifampicin to anti-enterococcal treatment regimens warrants further investigation and may prove useful in the treatment of enterococcal infections whilst prolonging the clinically useful life of currently active antibiotics.

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1. Introduction

Enterococci cause a range of infections from uncomplicated urinary tract infection to life-threatening endocardial and device-related infections. These pathogens are intrinsically resistant to a number of commonly used antimicrobials and have a remarkable ability to acquire new resistance mechanisms. This situation has fuelled global concern over future treatment options for serious enterococcal infections caused by multidrug-resistant strains.

Antibacterial agents commonly utilised or recently developed for the treatment of enterococcal infections include vancomycin, linezolid, daptomycin, telavancin and dalbavancin. Tigecycline has been suggested as an alternative therapy but, with the exception of intra-abdominal infections, a current lack of clinical data has impeded greater use [1]. As resistance to many of these antibacterials increases, including tigecycline [2], therapeutic options become progressively more limited and the need for strategies to protect against further loss of activity becomes paramount.

Prescribing antibacterial combinations is established clinical practice for the treatment of serious infections. There are several potential advantages to combined therapy: an enhanced killing effect and the possibility of synergy; a reduction in the concentration of individual agents required (reduced toxicity and selection pressure); and, of vital importance, the ability to protect against the development of resistance. For example, daptomycin is recommended as part of a combined therapy for serious infections [3,4]. Rifampicin is rapidly bactericidal against many Gram-positive bacteria and displays good tissue penetration, but the rapid development of

resistance precludes its use as monotherapy [4], leading to this old agent often being considered specifically for use in combination therapy [5,6].

The aim of this study was to describe the killing effect of different antibacterial combinations against clinical enterococcal isolates using standard methods and in an in vivo *Galleria mellonella* infection model.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

Eight enterococcal isolates were studied, including three vancomycin-sensitive strains [*Enterococcus faecalis* ATCC 29212 and *E. faecalis* clinical isolates E019 and E045] and four vancomycin-resistant strains [*E. faecalis* ATCC 51299, *Enterococcus faecium* ATCC 51559 and *E. faecium* clinical isolates E022 and E039] and one vancomycin-sensitive tigecycline-resistant *E. faecalis* UW6940 (supplied by Dr Werner, Centre for Diagnostic Medicine, Germany) (Table 1).

2.2. Preparation of antibiotics

Vancomycin and rifampicin were purchased from Sigma-Aldrich (Dorset, UK). Linezolid and tigecycline were gifted from Pfizer Ltd. (Surrey, UK). Antibiotic stocks of 10 000 mg/L (except linezolid, 1000 mg/L) were freshly prepared using distilled water each day (linezolid and tigecycline) or were stored at $-20\text{ }^{\circ}\text{C}$ for a maximum of 1 week (vancomycin and rifampicin).

2.3. Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) of antibiotics were determined by the broth microdilution method described by the British Society for Antimicrobial Chemotherapy (BSAC) for each of the eight isolates [7]. *Enterococcus faecalis* ATCC 29212 was included as a control strain in each experiment, and all results were within guideline limits. MIC determinations were performed in duplicate and were repeated on two further occasions.

2.4. Antibacterial combination assays

Standard chequerboard assays were performed in 96-well microtitre plates (Sterilin Ltd., Gwent, UK) with doubling dilutions of each antibiotic prepared in Mueller–Hinton broth (Oxoid Ltd., Basingstoke, UK). The final sub-MIC ranges used for vancomycin, rifampicin, tigecycline and linezolid were 0.12–1024, 0.12–32, 0.007–4 and 0.12–8 mg/L, respectively. An equal volume of standardised bacterial suspension of 5×10^5 CFU/mL was added and the plates were incubated at 37 °C in air for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of drug A or B in combination divided by the MIC of drug A or B alone, respectively, and the FIC index (FICI) was obtained by adding the two FIC values. The drug combination that consistently generated the lowest FICI after repeating the experiment in duplicate on two further occasions was used to categorise the results as follows: FICIs of ≤ 0.5 were interpreted as synergistic; FICIs of >0.5 but <4 were considered as no interaction; and FICIs ≥ 4 were interpreted as antagonistic [8]. Combinations demonstrated to be synergistic were assessed using the *G. mellonella* infection model.

2.5. *Galleria mellonella* infection model

Galleria mellonella wax moth larvae (Livefood UK Ltd., Rooks Bridge, UK) in their final instar stage of development were stored at room temperature in the dark and were used within 1 week of delivery. Healthy larvae, without grey markings and of a similar weight (200–300 mg), were selected and split into experimental groups of 15 larvae [9]. Bacterial suspensions of each isolate were prepared based on a pre-optimised dose (1×10^6 – 5×10^6 CFU/larva) that caused >80% larvae deaths at 72 h post-infection (Supplementary Fig. S1).

After sterilisation of the inoculation site with 70% (v/v) ethanol, the last right proleg was used to deliver 10 μ L of bacterial suspension (as recorded in Fig. 1) into the haemocoel (primary body cavity) using a 1/2 inch, 30 G needle (BD Precisionglide[®] syringe needle; Sigma) attached to a 50 μ L Hamilton syringe (1705 TLL; Jaytee Biosciences Ltd., Herne Bay, UK). Antimicrobial drugs (used at $1 \times$ MIC as monotherapy or between $1/4 \times$ MIC and $1/16 \times$ MIC for antibacterial combinations) were delivered into the haemocoel via a 10 μ L injection into the last left proleg ($n = 15$). Larvae were incubated in vented plastic Petri dishes at 37 °C in air and deaths were scored through observation of melanisation and failure of larvae to move in response to touch at the time points 12, 24, 48, 72 and 96 h. Appropriate uninfected and vehicle controls were included for each experiment.

Pooled data from three independent experiments, using *G. mellonella* larvae obtained from different batches, were assessed using the Kaplan–Meier method,

and treatment groups were compared by the log-rank (Mantel–Cox) test using GraphPad Prism[®] 6 (GraphPad Software Inc., La Jolla, CA). *P*-values of <0.05 were considered statistically significant.

3. Results

3.1 Assessing the *in vitro* antimicrobial sensitivities of eight enterococcal isolates using the chequerboard dilution method

The results of susceptibility testing are shown in Table 1. No antimicrobial combination showed an antagonistic effect against any of the strains evaluated. Of the six antimicrobial combinations tested against eight strains, synergy was seen in six cases, but in each case it was a rifampicin-containing combination. Synergy was demonstrated against all vancomycin-resistant enterococci by at least one rifampicin-containing antibacterial combination (all *E. faecium* isolates and one *E. faecalis* isolate) (Tables 1 and 2).

3.2. Antimicrobial treatment of infected *Galleria mellonella* larvae

Dose-dependent killing of *G. mellonella* was achieved when larvae were infected with 1×10^6 , 3×10^6 or 5×10^6 CFU/larva of each isolate. *Enterococcus faecalis* ATCC 51299 was highly virulent at all three doses tested, whilst *E. faecium* ATCC 51559, *E. faecium* E022 and *E. faecium* E039 were less virulent in the *G. mellonella* model (Supplementary Fig. S1).

Treatment of infected larvae with an antibacterial combination of sub-MIC agents consistently led to an increased level of survival (20–73% larvae survival at 96 h,

with a median value of 57%) compared with those treated with a higher concentration of a single agent (7–53% survival, with a median value of 13%) (Fig. 1). A statistically significant greater level of survival was observed with four of the six combinations tested compared with the untreated control group (Fig. 1a,c,e,f), whilst only two vancomycin monotherapies administered at 1× MIC significantly improved survival of *G. mellonella* compared with the control group (Fig. 1c; $P = 0.003$; Fig. 1e, $P = 0.0159$). Furthermore, the combination of rifampicin with tigecycline was statistically superior to tigecycline alone (13% larval survival when treated with tigecycline for infection with strain E039 vs. 60% larval survival when treated with the antimicrobial combination; $P = 0.0237$) (Fig. 1f).

4. Discussion

Antibacterial combinations are often utilised during treatment of serious infections, but the superiority of one antibiotic combination over another for the treatment of enterococcal infections remains unproven. In this study, it was demonstrated that antimicrobial combinations including rifampicin can be synergistic against enterococci, but this is not a consistent finding among enterococci.

Although traditionally used in the investigation of antibacterial combinations for synergistic activity, checkerboard assays have reproducibility issues and may not adequately reflect activity in vivo. The in vivo *G. mellonella* infection model, however, shares some basic immunity characteristics with mammals, including the deployment of proteolytic cascades (clotting and melanisation) following pathogen recognition [10]. An additional advantage, and in contrast to mammalian models, *G. mellonella* can be inexpensively sourced and is not subject to animal research legislature [11].

In this study, the larval model corroborated the in vitro data. Treatment with antibacterial combinations that were synergistic in vitro improved the survival rate of larvae compared with those treated with a single agent.

All combinations where synergy was detected were performed with concentrations of antibacterial below the MIC ($1/4\times$ to $1/16\times$ MIC) and contained rifampicin, with rifampicin with either vancomycin or tigecycline being the most effective combinations. Of the five vancomycin or tigecycline combinations trialled, a statistically greater proportion of the larvae treated with combination therapy survived in four of the assays compared with wax moths that were either untreated or received only one antibiotic.

Rifampicin is effective against a range of Gram-positive pathogens, but the rapid development of resistance necessitates that it be used in combination with another agent. Several studies have highlighted the synergistic activity of rifampicin with others agents [6,12]; indeed, rifampicin-based combinations, including vancomycin, are recommended for the treatment of staphylococcal endocarditis [3]. Combinations incorporating rifampicin are not, however, in routine use for treatment of enterococcal infections.

The classic combination of a cell-wall-active agent (such as vancomycin or a β -lactam) plus an aminoglycoside results in a synergistic effect against enterococci [13]. High-level aminoglycoside resistance has, however, led to a reconsideration of this standard treatment (gentamicin and ampicillin) resulting, in some instances, in

the recommendation of an unusual double β -lactam combination (ampicillin plus ceftriaxone) [14,15]. In addition, the continual rise of vancomycin resistance has limited the value of this agent in classic treatment and, as a consequence, excluded it in many instances from combination therapy studies. Yet a vancomycin-based combination has shown efficacy against resistant enterococci [16]. In the current study, a vancomycin plus rifampicin combination improved the survival rate of *G. mellonella* larvae infected with selected rifampicin- and/or vancomycin-resistant enterococcal strains.

Typically, the interaction of tigecycline with other agents results in indifference or occasionally antagonism, an exception being with rifampicin [17]. High rates of in vitro synergism have been described for tigecycline plus rifampicin against *E. faecalis* and *E. faecium* isolates using the checkerboard dilution method [5,18]. More recently, Silvestri et al. tested tigecycline plus rifampicin combinations in an animal model of surgical wound infections and reported good activity against enterococcal isolates [19], supporting the observations reported here with the wax moth infection model.

The failure to assess any reduction in susceptibility to agents during treatment was a limitation of the current study, since the rapid development of resistance against rifampicin in particular will always remain a concern and the observations of Holmberg and Rasmussen indicate that combined therapy might not be sufficient to prevent this from developing [20].

In conclusion, the study has revealed the efficacy of rifampicin-based combination therapies against some highly-resistant enterococci, and further investigation in vivo with additional clinically relevant strains is warranted.

Antibiotic combinations offer the potential to treat problematic antimicrobial-resistant bacteria with lower concentrations of antibiotic without compromising efficacy and with a lower risk of adverse side effects. Moreover, combination therapy has the potential to reduce selective pressure and to help protect the clinical life of agents, particularly newer agents for which little, if any, resistance exists. In addition, the checkerboard assay remains a useful screening tool for the detection of potentially synergistic antimicrobial combinations, with the wax moth model being a practical and superior technique for providing quantitative in vivo data.

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Fig. 1. Effect of antibiotic treatment on survival of *Galleria mellonella* larvae infected with (a) *Enterococcus faecalis* ATCC 51299, (b) *Enterococcus faecium* ATCC 51559, (c,d) *E. faecium* E022 and (e,f) *E. faecium* E039 ($n = 45$). MIC, minimum inhibitory concentration; VAN, vancomycin; RIF, rifampicin; LZD, linezolid; TGC, tigecycline. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ for tested antibiotic combinations compared with the untreated control; ^ $P < 0.05$ for tested antibiotic combinations compared with tigecycline alone.

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Table 1. Standard antibiotic susceptibility and antibacterial combination results for eight enterococcal isolates

Enterococcal isolate	MIC (mg/L) ^a				Lowest FICI generated by each drug combination ^b					
	VAN	RIF	LZD	TGC	VAN + TGC	VAN + RIF	TGC + RIF	LZD + VAN	LZD + TGC	LZD + RIF
<i>E. faecalis</i> ATCC 29212	2	2	2	0.12	0.62	0.62	0.62	1	1	0.62
<i>E. faecalis</i> E019	1	4	2	0.25	1	1	0.75	1	1	0.62
<i>E. faecalis</i> E045	2	1	2	0.12	0.75	0.75	0.75	1.5	1	0.75
<i>E. faecalis</i> ATCC 51299	64	1	1	0.12	0.62	0.37	0.75	0.75	0.75	0.62
<i>E. faecalis</i> UW6940	1	2	1	2	0.75	1	0.75	1	0.75	0.75
<i>E. faecium</i> ATCC 51559	256	8	2	0.12	1	0.75	0.62	0.56	0.75	0.5
<i>E. faecium</i> E022	512	4	1	0.12	0.62	0.37	0.5	0.56	0.56	0.62
<i>E. faecium</i> E039	512	16	2	0.12	0.75	0.31	0.37	0.62	0.75	0.62

MIC, minimum inhibitory concentration; VAN, vancomycin; RIF, rifampicin; LZD, linezolid; TGC, tigecycline; FICI, fractional inhibitory concentration index.

^a Antibiotic susceptibility breakpoints: VAN, RIF and LZD, sensitive ≤ 4 mg/L, resistant >4 mg/L; TGC, sensitive ≤ 0.25 mg/L, intermediate 0.5 mg/L, resistant >0.5 mg/L.

^b Impact of antibacterial combinations: synergy, FICI ≤ 0.5 (shown in bold); no interaction, FICI >0.5 to <4 ; and antagonism ≥ 4 .

Table 2. Enterococcal strains displaying synergy with antibiotic combinations

Enterococcal isolate	Antibiotic (drug A) synergistic combination with RIF	MIC of drug A and RIF alone (mg/L)		Concentration of the drug in combination (mg/L)		FIC		FICI
		Drug A	RIF	Drug A	RIF	Drug A	RIF	
<i>E. faecalis</i> ATCC 51299	VAN	64	1	16	0.12	0.25	0.12	0.37
<i>E. faecium</i> ATCC 51559	LZD	2	8	0.5	2	0.25	0.25	0.5
<i>E. faecium</i> E022	VAN	512	4	64	1	0.125	0.25	0.37
	TGC	0.12	4	0.03	1	0.25	0.25	0.5
<i>E. faecium</i> E039	VAN	512	16	128	1	0.25	0.06	0.31
	TGC	0.12	16	0.03	2	0.25	0.12	0.37

RIF, rifampicin; VAN, vancomycin; LZD, linezolid; TGC, tigecycline; MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.