Synergistic activity of fosfomycin and chloramphenicol against vancomycin-resistant Enterococcus faecium (VREfm) isolates from bloodstream infections

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Highlights

- Vancomycin-resistant enterococci (VRE) antibiotic options are limited
- Fosfomycin maintains good efficacy against VRE but clinical experience is limited. VRE susceptibility to chloramphenicol is ≥ 90%.
- We tested the combination of chloramphenicol + fosfomycin (CAF+FOS) against 10 VRE strains from bloodstream infections
- CAF+FOS combination determined a synergic effect observed for 50% of the isolates and an additive effect for the remaining 50%
- The survival of *G. mellonella* larvae infected with a lethal dose of a VRE strain was significantly higher with antibiotic combination
Synergistic activity of fosfomycin and chloramphenicol against vancomycin-resistant *Enterococcus faecium* (VREfm) isolates from bloodstream infections

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**ABSTRACT**

Vancomycin-resistant *Enterococcus faecium* (VREfm) infections are increasing. Current anti-VREfm options (linezolid and daptomycin) are suboptimal. Fosfomycin maintains good efficacy against VREfm and chloramphenicol is active against \(\geq 90\%\) of VREfm. We tested chloramphenicol + fosfomycin (CAF+FOS) against 10 VREfm isolated from blood. MICs
were 64-512 µg/ml for fosfomycin and 8-16 µg/ml for chloramphenicol. The combination decreased both MICs, with a synergic effect in 50% of the isolates and an additive effect in the remaining 50%. Time-kill assays performed on FICI ≤ 0.5 strains confirmed the synergism. The antibiotic combination at ¼ MICs caused a ≥ 2 log₁₀ reduction compared to the two antibiotics alone. Finally, we provided a proof of concept of the in vitro efficacy of CAF+FOS in G. mellonella. The survival of G. mellonella larvae treated with the combination was significantly higher. The activity of fosfomycin and chloramphenicol against VREfm increases when they are used in combination.

KEYWORDS

Chloramphenicol; fosfomycin; Galleria mellonella; synergism; VRE

1. INTRODUCTION

Epidemiology of vancomycin-resistant E. faecium (VREfm) infections is continuously changing with significant differences in incidence trends between countries [1–3]. In Europe and United States (US) approximately one third of healthcare-associated enterococcal infections and 10% of bloodstream enterococcal infections are caused by vancomycin resistant strains [4–6].

In 2018, the World Health Organization has included VREfm in the priority list of antibiotic-resistant bacteria, ranking 12th in the priority urgency, just before methicillin-resistant Staphylococcus aureus [7].

Clinical outcomes associated with VREfm infections are more severe than historically expected: vancomycin resistance is a known risk factor for mortality in patients with
enterococcal bloodstream infections with a nearly double mortality observed in VREfm bacteremias compared to vancomycin-susceptible *Enterococcus* bacteremias [8].

Although it is likely that the mortality risk of VREfm bacteremic patients is mainly attributable to their underlying comorbidities and illness severity, issues regarding current anti-VRE antibacterial options exist. In fact, both the first two line drugs against VREfm bacteremia retain several caveats: 1) linezolid, bacteriostatic and lipophilic, is not the *optimum* for bloodstream infections and, 2) daptomycin efficacy carries several dose-related concerns [9], it is often used in combination with a beta-lactam (e.g. ampicillin, ceftaroline) and EUCAST breakpoints are not available.

Chloramphenicol is a broad-spectrum antibiotic acting by inhibiting bacterial protein synthesis. It has been nearly dismissed in western countries for potential myelotoxicity but VREfm susceptibility to this antibiotic has been demonstrated to be $\geq 90\%$ [10].

Fosfomycin is a small (138 g/mol) bactericidal antibiotic that acts by inhibiting the first step of bacterial cell wall biosynthesis [11]. Fosfomycin maintains good efficacy *in vitro* against VREfm isolates but clinical experience is almost limited to urinary tract infections [12].

The aim of our study was to assess the *in vitro* and *in vivo* effectiveness of chloramphenicol in combination with other antibiotics, particularly with fosfomycin, against clinical VREfm isolates collected from patients with bloodstream infections.

2. METHODS

2.1 Bacterial strains and culture conditions

The study was conducted on ten nonduplicate *E. faecium* isolates collected from bloodstream infections of patients admitted to the Trieste University hospital. Microbial identification and antimicrobial susceptibility testing were routinely performed using the VITEK2 automated
The isolates were investigated for the presence of the vanA and vanB determinants by PCR, using specific primers (A1: 5’-GGGAAAACGACAAATGC-3’ and A2: 5’-GTACAATCGGCGGTTA-3’ for vanA; B1: 5’-ATGGGAAGCCGATAGTC-3’ and B2: 5’-GATTTCGTTCTCGACC-3’ for vanB) and conditions previously described [13]. Confirmatory MIC testing for chloramphenicol and fosfomycin was carried out by the microdilution method in Muller Hinton broth (MHB), supplemented with 25 µg/ml glucose-6-phosphate (G6P) for testing of fosfomycin, and interpreted according to the Clinical and Laboratory Standards Institute guidelines [14]. *E. faecalis* ATCC 29212 was used for quality control in antimicrobial susceptibility testing.

### 2.2 Synergy testing by checkerboard assay

Five antibiotics were tested for synergy with chloramphenicol, including fosfomycin, linezolid, daptomycin, nitrofurantoin and vancomycin. All drugs were purchased from Merck KGaA (Darmstadt, Germany). For each of them the concentration range for checkerboard assay was determined by preliminary evaluation of susceptibility of the different strains by microbroth dilution. Synergy testing was carried out in 96-well microtiter plates, on an initial inoculum of 5x10^5 cfu/ml, using a 7-by-5 well configuration. The plates were incubated for 24 h at 37°C. Interactions between antibiotics were determined by calculating the fractional inhibitory concentration index (FICI), according to the following formula: MIC of drug A in combination/MIC of drug A acting alone + MIC of drug B in combination/MIC of drug B acting alone. Data were obtained from at least two independent experiments. Results were interpreted as follows: FICI ≤ 0.5, synergism; 0.5 < FICI ≤1, additive effect; 1 < FICI ≤ 4, no interaction; and FICI > 4, antagonism [15].
2.3 Time-kill assay

Time-kill study was conducted at ¼ MIC to confirm the synergism detected by checkerboard assay. Bacterial strains of interest were inoculated at 5 x 10^5 cfu/ml in 5 ml tubes containing the drugs alone and in combination. One tube without drug was included as growth control in each experiment. Viable cells were evaluated by plating serial dilutions at 0, 4, 8 and 24 h. Time–kill curves were generated by plotting mean colony counts of three independent experiments (log_{10} cfu/ml) ± standard deviation. Results were interpreted as follows. The difference in cfu/ml at 24 h between the combination tube and the most active antibiotic alone was calculated. If this difference was ≥2log_{10} the combination was considered synergistic. Furthermore, the difference between the cfu/ml detected at 24 h and those inoculated at time 0 was calculated for each sample; considering bactericidal the combinations that caused a ≥3 log_{10} cfu/ml reduction [15].

Finally, the antibiotic combination showing the highest synergistic activity was selected for in vivo testing.

2.4 Galleria mellonella infection and treatment assays.

G. mellonella larvae were purchased from the Serpens breeding (Paliano, Italy; www.bigserpens.com) and used within one day of shipment. The average weight of larvae was about 400 ± 50 mg. To determine the infective dose of E. faecium Ef-10 required to kill G. mellonella larvae, the strain was grown in MHB until mid-exponential phase, and serial ten- or three-fold dilutions of bacterial cell suspensions in saline were injected into G. mellonella larvae in the second-last left proleg. The actual number of bacterial cells injected into the larvae was determined by serial dilution and plating. Infected larvae were incubated at 37°C for up to 3 days to monitor mortality. The number of larvae used and the number of
replicates performed for each experiment are indicated in the corresponding figure legend. Each replicate also included larvae infected with saline, to verify the impact of physical trauma, and untreated larvae to control their healthy state. Kaplan-Meier survival curves, lethal doses 50 and 90% (LD$_{50}$ and LD$_{90}$) and R$^2$ values were obtained using GraphPad Prism as described [16].

The in vivo antibacterial activity of fosfomycin and chloramphenicol alone or in combination was evaluated in G. mellonella larvae infected with a dose of E. faecium Ef-10 close to the LD$_{90}$. At 20 minutes post-infection, 10 µl of water solutions of fosfomycin alone (at 6.25, 12.5 or 25 mg/mL), chloramphenicol alone (at 0.625, 1.25 or 2.5 mg/mL), or the combination of both antibiotics (at 6.25 and 1.25 for fosfomycin and chloramphenicol, respectively) were injected into the second-last right proleg. The control group was infected as described above and treated with 10 µl of water. Larvae were incubated at 37°C and survival was monitored for 3 days.

3. RESULTS

3.1 Evaluation of susceptibility to the different antibiotics alone and combined with chloramphenicol

The ten VREfm isolates were initially investigated by PCR amplification to identify their vancomycin resistance determinant, revealing an equal distribution of the vanA and vanB determinants. As expected, the five vanA isolates showed a higher MIC for vancomycin than those carrying vanB (Table1).

Before investigating the activity of chloramphenicol combined with 5 different drugs, the susceptibility in MHB for each antibiotic alone was evaluated. Towards chloramphenicol, 2 of the tested isolates showed an intermediate susceptibility level (MIC=16 µg/ml) and 8 were
susceptible, with MICs exactly corresponding to the breakpoint (8 µg/ml). For the other antibiotics, MICs varied above and below the CLSI breakpoints, with 4 isolates resistant to daptomycin, 5 intermediate/resistant to linezolid, 3 intermediate to nitrofurantoin and 7 resistant to fosfomycin. When tested by checkerboard assay, chloramphenicol in combination with fosfomycin showed synergistic on 5 out of 10 isolates and additive on the other 5, with the MIC of fosfomycin lowered below the clinical breakpoint in all but one isolate. Combinations with other antibiotics looked less effective, being mainly additive. Synergism was only detected only in one isolate for the combination with daptomycin or vancomycin. Anyway, antagonism was not observed in our study. MIC values of the different antibiotics, alone and in combination, and the relative FIC indexes are presented in Table 1.

Since chloramphenicol + fosfomycin (CAF+FOS) was the antibiotic combination with the highest synergistic activity against the largest number of isolates (Table 1), it was further investigated in the following experiments.

3.2 Time-kill analysis of the combination CAF+FOS

The two antibiotics used separately at ¼ MIC showed different activity. Chloramphenicol produced a growth slowdown during the first 8 hours incubation, followed by a complete rebound at 24 h. Fosfomycin exerted a bacteriostatic effect at the beginning of incubation, followed, from 8 h onwards, by the recovery of growth ability, although the population density remained a little lower (0.5-1 log_{10}) compared to the control, even after 24 h incubation. This assay confirmed the synergism of the combination CAF+FOS, as it caused a final reduction ≥2 log_{10} cfu/ml than the most active agent alone (in this case fosfomycin) in all the tested strains. Moreover, for each treatment the number of viable cells was evaluated
at 24 h and compared to the initial inoculum, showing a reduction \(< 3 \log_{10}\), which corresponds to a bacteriostatic effect. (Figure 1).

3.3 In vivo G. mellonella assays

To provide a proof of concept that the CAF+FOS combination can also work in vivo for the treatment of VREfm infections, we used the G. mellonella infection model, which has been previously exploited to validate novel antibiotic combinations against VREfm [17–19]. Preliminary experiments were performed to assess the infectivity of the VREfm strain chosen for the analysis (Ef-10) in G. mellonella larvae and to determine the dosages of fosfomycin and chloramphenicol that were effective in reducing Ef-10 infectivity when administered alone. In our experimental setting, the Ef-10 strain had a LD\(_{90}\) of about 4×10\(^6\) CFU (Figure S1), and fosfomycin and chloramphenicol alone were able to significantly decrease the lethality caused by a lethal dose of Ef-10 at \(\geq 313\) mg/kg or 62.5 mg/kg, respectively (Figure S2). To evaluate the in vivo efficacy of the CAF+FOS combination, Ef-10-infected G. mellonella larvae were therefore treated with fosfomycin at 156 mg/kg, chloramphenicol at 31.2 mg/kg, or the combination of both antibiotics at the same concentrations. As shown in Figure 2, CAF+FOS almost completely abrogated the lethality caused by Ef-10 infection in G. mellonella larvae. Moreover, the antibiotic combination was significantly more effective than each antibiotic alone, even if in these infection assays also fosfomycin at 156 mg/kg caused a slight, significant increase in G. mellonella survival (about 20%) with respect to the chloramphenicol alone and control groups (Figure 2).
4. DISCUSSION

The combination CAF+FOS, preliminary investigated in vitro and then validated by an in vivo proof of concept, showed efficacy against VREfm clinical isolates from bloodstream infections, determining an increase in the susceptibility to both antibiotics in all the tested strains. The combination, either acting in a synergistic or in an additive mode, lowered the fosfomycin MIC values below the breakpoint for all but one the resistant/intermediate isolates.

Chloramphenicol and fosfomycin are quite different both in terms of both pharmacokinetic and pharmacodynamic properties. Fosfomycin is a small hydrophilic bactericidal molecule acting on the bacterial cell wall while chloramphenicol is a lipophilic bacteriostatic molecule acting on bacterial protein synthesis. Intravenous fosfomycin is almost invariably administered as partner drug to take advantage of its wide synergistic potential [20,21]. Contrary to the past beliefs, chloramphenicol safety has been demonstrated to be comparable to alternative antibiotics, especially for brief therapies [22] and fosfomycin has an excellent safety profile [23]. Both chloramphenicol and fosfomycin have been placed in the WHO model list of essential medicines [24]. Moreover, both antibiotics share a good central nervous system penetration that makes the combination CAF+FOS attractive for emerging VREfm infections such as post-surgical or shunt-associated meningitis [25].

In conclusion we demonstrated that the combination CAF + FOS was synergic (50%) and additive (50%) against VREfm clinical strains isolated from bloodstream infections. In addition, such combination was more effective than each antibiotic alone in increasing survival in a *G. mellonella* infection model. These data support to carry out further clinical investigations to evaluate CAF + FOS combination therapy in VREfm infections.
Authors’ statement
We declare no conflict of interest

Authors’ contributions
Cristina Lagatolla, Johana Milic and Raffaela Bressan performed the in vitro experimental study, Francesco Imperi and Matteo Cervoni performed the animal experimental study, Roberto Luzzati wrote the conclusions paragraph and reviewed the manuscript, Stefano Di Bella conceived the experimental idea and wrote the manuscript

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Competing interests: None declared.

Ethical approval: Not required
Figure 1: Twenty-four hours time-kill curves of chloramphenicol and fosfomycin, alone and in combination, at ¼ of their MIC values on five VREfm strains for which a FICI ≤ 0.5 was obtained by checkerboard assay. Abbreviations: CAF, chloramphenicol; FOS, fosfomycin.
Figure 2. Kaplan-Meier survival curves of *G. mellonella* larvae infected with 5.0(±2.4)×10^6 CFU of *E. faecium* Ef-10 and then treated with 31.2 mg/kg of chloramphenicol (CAF) or 156 mg/kg fosfomycin (FOS) alone or the combination of both antibiotics at the same concentrations (FOS+CAF). The control group was treated with water. Forty larvae were used for each group in four different experiments. Asterisks highlight statistically-significant differences (*P*<0.05, Mantel-Cox test).

References


<table>
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<th>Strain (VAN(^\text{determinant}))</th>
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<th>LZD</th>
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<th>NIT</th>
<th>VAN</th>
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\(a\) Antibiotic abbreviations: CAF, chloramphenicol; FOS, fosfomycin; LZD, linezolid; DAP, daptomycin; NIT, nitrofurantoin; VAN, vancomycin.

\(b\) MIC values above the clinical breakpoints are highlighted in grey.

\(c\) Synergisms are highlighted in green.