In Vitro Antimicrobial Activity of Fosfomycin, Rifampin, Vancomycin, Daptomycin Alone and in Combination Against Vancomycin-Resistant Enterococci

Wei Yu
Zhejiang Provincial People's Hospital https://orcid.org/0000-0002-1174-596X
Yiheng Jiang
Nanjing Medical University
Hao Xu
Zhejiang University
Li Zhang
Zhejiang University
Xuehang Jin
Zhejiang University
Shuaibin Ying
Zhejiang University
Yunqing Qiu (qiuyq@zju.edu.cn)
Zhejiang University

Research

Keywords: vancomycin, daptomycin, combination therapy, biofilm

Posted Date: February 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-170303/v1

Abstract

OBJECTIVES

The emergence of vancomycin resistant enterococci (VRE) is shortening the choices for clinical anti-infective therapy. The aim of this study was to investigate the mechanism of vancomycin resistance and evaluate the effect of fosfomycin (FM), rifampin (RIF), vancomycin (VAN), linezolid (LNZ), daptomycin (DAP) alone or in combination against VRE.

METHODS

Eight VRE isolates were collected. A total of 18 antibiotics susceptibility tests were further done for VRE. Whole genome sequencing and bioinformatics analysis were performed. The effect of FM, RIF, VNA, LNZ, DAP alone or in combination was determined using anti-biofilm testing and the time-kill assay.

RESULTS

All isolates were susceptible to LNZ and DPA. The high-level resistance determinant of VAN in these strains was due to VanA-type cassette. MLST revealed two different STs for vancomycin-resistant *Enterococcus faecium* (VREm) and four different STs for vancomycin-resistant *E. faecalis* (VREs). Virulence genes in VREs were more than VREm, especially for 4942 isolated from blood. Gene *acm* and *uppS* were only identified in VREm, while virulence genes related to cytolysin were only found in E. faecalis. Further *in vitro* anti-biofilm testing and time-kill assay found FM (83 mg/L) combined with DAP (20.6 mg/L) and DAP monotherapy (47.1 mg/L) showed bactericidal effect against 8 tested VRE strains at 24h.

CONCLUSIONS

The high-level resistance determinant of VAN in these strains was due to VanA-type cassette. FM combined with DAP might be greater potential therapeutic option against VRE.

1. Introduction

Vancomycin resistant enterococci (VRE) are increasingly becoming public health threat for hospitals worldwide. The opportunistic invasive infections caused by VRE are often broadly resistant to available antibiotics. In addition, the spread of VRE is difficult to control due to longer periods of VRE on inanimate surfaces [1]. Previous studies reported VRE caused significant mortality, ranging from 19–63% [2–3]. The two most prevalent and clinically relevant *Enterococcus* species are vancomycin-resistant *Enterococcus faecium* (VREm) and *E. faecalis* (VREs) [4]. In previous years, most VRE infections were caused by *E. faecalis* [5]. However, since 2002 an increase in the prevalence of VREm has been observed, with reports of VREm being as common as VREs in 2006 [6]. This could be due to intrinsic and acquired resistance to many classes of antibiotics of *E. faecium*, making it better adapted to the hospital and environment where antibiotic use is common [7]. Although *E. faecalis* also exhibits intrinsic and acquired resistance to a variety of antibiotic classes, the presence and level of resistance can differ between species [8].

Currently considered effective treatments are quinupristin/dalfopristin, tigecycline, teicoplanin, telavancin, linezolid (LNZ), and daptomycin (DAP) [9]. However, VRE continues to cause significant mortality and resistance arises. There are relatively few studies in this important area of combination treatment with synergistic antibiotics. Thus, to elucidate the *in vitro* effectiveness, we compared the antibacterial effects of fosfomycin (FM), rifampin (RIF), vancomycin (VAN), LNZ, DAP alone and in combination against VRE.

2. Methods

2.1. Bacterial strains and antibiotic susceptibility test

A total of 8 VRE strains were isolated from clinical specimens and identified using Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) (Bruker Diagnostics, Bremen, Germany).

The minimum inhibitory concentrations (MICs) for 18 antibiotics, including oxacillin, penicillin, meropenem, erythromycin, clindamycin, trimethoprim-sulfamethoxazole, amikacin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline, tigecycline, RIF, VAN, LNZ, FM, and DAP were determined by broth microdilution [10]. The susceptibility to FM was tested by agar dilution. The media was supplemented with 50 mg/l Ca^{2+} for testing of DAP and 25 mg/l glucose-6-phosphate (G6P) for FM. *E. faecalis* ATCC 29212 was used as quality control.

2.2. Whole-Genome Sequencing (WGS)

WGS was carried out for 8 VRE isolates with further analyses of gene-environment. Genomic DNA was extracted by FastDNA SPIN Kit for Soil (MP Biomedicals, United States) and sequenced using HiSeq 2000 (Illumina, SanDiego, CA, USA) with constructing 2x125-bp pair-end libraries. De novo assembly was done using the CLC Workbench v8.0 (QIAGEN, Hilden, Germany). The resistance genes, and virulence genes were identified by BLAST against the ResFinder 2.1 database (https://cge.cbs.dtu.dk/services/ResFinder/). The bioinformatics tools used in this study were available at the following web platforms: NCBI (National Center for Biotechnological Information), SMS (Sequence Manipulation Suite), and EBI (European Bioinformatics Institute).

This Whole Genome Shotgun BioProject for VRE has been deposited at GenBank under the accession PRJNA662846 and PRJNA662849 (Supplemental Table 1).

2.3. Anti-biofilm testing

All VRE isolates and ATCC 29212 were inoculated into 96-well polystyrene microtiter plates with Mueller–Hinton II broth (MHB) and different RIF, LNZ, FM, and DAP concentration for 24h, 48h, and 72h to test the biofilms formation as a previous study [11].

2.4 Anti-complement killing test

Serum was collected from healthy mice and centrifuged to obtain normal mouse serum. This was placed in a water bath at 56°C for 30 min to inactivate complement, generating inactive serum. An overnight bacterial culture was diluted to a cell density of 2×10^6 CFU/mL, and normal and inactivated sera (180 µL) were separately mixed with 20 µL bacterial suspension and incubated at 37°C for 1 h. Samples were diluted 100-fold, spread onto plates, and incubated overnight, and colonies on plates were counted. The bacterial survival rate was calculated using the following formula:

Bacterial survival rate = (number of colonies with normal serum/number of colonies with inactivated serum) × 100%.

ATCC 29212 served as the control strain.

2.5 Time–kill assays

The bactericidal activities of FM, VAN, and DAP alone or in combination against four VRE (4942, 12022, 19372, 23760) and ATCC 29212 were investigated using the time–kill method [12]. The following concentrations were used: FM 83 mg/L [13]; LNZ 10 mg/L [14]; RIF 3 mg/L [15]; VAN 13.3 mg/L [16]; DAP 20.6 mg/L, 31.1 mg/L and 47.1 mg/L [17]; FM 83 mg/L + DAP 20.6 mg/L; FM 83 mg/L + LNZ 10 mg/L; FM 83 mg/L + RIF 3 mg/L; FM 83 mg/L + VAN 13.3 mg/L; RIF 3 mg/L + DAP 20.6 mg/L; RIF 3 mg/L + LNZ 10 mg/L; RIF 3 mg/L + VAN 13.3 mg/L. The media was supplemented with 25 mg/l G6P for testing of FM and 50 mg/l Ca^{2+} for DAP.

3. Results

3.1 Antimicrobial susceptibility and multi-locus sequence typing (MLST)

There were two E. faecalis isolates and six E. faecium isolates included in this study. Eight VRE isolates obtained from culture including urine (n = 3), bile (n = 1), and blood (n = 4). The eight VRE isolates were all high-level resistance, whereas they were susceptible to tigecycline, LNZ, DPA (Table 1). Only two E. faecalis isolates (4942 and 12022) were susceptible to RIF.

Table 1 Minimum inhibitory concentrations of 18 antimicrobial agents against 8 VRE isolates

Antibiotics	4942 (*E. faecalis*)	5057 (*E. faecium*)	5173 (*E. faecium*)	5743 (*E. faecium*)	9604 (*E. faecium*)	12022 (*E. faecalis*)	19372 (*E. faecium*)	23760 (*E. faecium*)
OXA	> 32	> 32	> 32	> 32	> 32	16	> 32	> 32
PEN	8	> 32	> 32	> 32	> 32	4	> 32	> 32
MEM	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
ERY	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
CLI	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
SXT	0.016/0.304	> 8/152	0.064/1.216	> 8/152	> 8/152	0.016/0.304	2/38	> 8/152
AMK	> 128	128	128	> 128	> 128	> 128	32	128
GEN	> 16	4	> 16	> 16	> 16	> 16	4	> 16
CIP	> 16	> 16	> 16	> 16	> 16	0.5	> 16	> 16
LVX	16	> 16	> 16	> 16	> 16	1	> 16	> 16
MFX	8	32	32	32	32	0.25	32	16
TC	32	64	64	16	4	32	16	64
TGC	0.25	0.125	0.25	0.25	0.125	0.25	0.25	0.125
RIF	1	8	8	2	4	0.5	4	4
VAN	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
LNZ	2	2	1	2	1	2	2	2
FM	> 512	128	128	256	128	> 512	64	128
DAP	1	2	2	2	2	0.5	2	2
Source	blood	blood	blood	blood	bile	urine	urine	urine
MLST	4	412	412	564	78	179	17	78

OXA, oxacillin; PEN, penicillin; MEM, meropenem; ERY, erythromycin; CLI, clindamycin; SXT, trimethoprim-sulfamethoxazole; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; TC, tetracycline; TGC, tigecycline; RIF, rifampin; VAN, vancomycin; LNZ, linezolid; FM, fosfomycin; DAP, daptomycin.

MLST revealed two different STs for VREs isolates 4942 (ST4), 12022 (ST179). There were four STs for VREm 5057, 5173 (ST412), 5743 (ST564), 9604, 23760 (ST78), 19372 (ST17).

3.2 Resistance genes and virulence genes

The isolate 23760 has the least number of resistance genes. Three genes *vanRA*, *vanSA*, *vanYA* were found in all isolates. VanA, VanXA were not found in 12022 and 23760. In addition, *vanHA* was not identified in 12022 and *vanZA* was not found in 23760 (Supplemental Table 2). The isolate 5734 had genomic island including *vanZ*, *vanY*, *vanX*, *vanA*, *vanH*, *vanS*, *vanR* (Supplemental Fig. 1).

Virulence genes *bopD* and *efaA* were found in all six isolates. Notably, virulence genes in VREs were more than VREm, especially for 4942 isolated from blood (Supplemental Table 3). Gene *acm* and *uppS* were only identified in VREm, while virulence genes related to cytolysin were only found in VREs.

3.3 Anti-complement killing test and anti-biofilm formation test

The survival rate of 5057 was less than 30%, while the rate of 5173 and 9604 were above 90%. (Supplemental Fig. 2).

As shown in Fig. 1, the biofilm formations of isolate 5057, 5173, 5743, 9604 were less than other four VRE isolates. Except for isolate 19372, FM monotherapy could not efficiently inhibit the formation of biofilms. LNZ and DAP monotherapy showed effective anti-biofilm formation during 72 hours. The biofilm inhibitory effect against planktonic VRE isolates for FM combined with LNZ or VAN was better than monotherapy. In addition, the effect of FM (83 mg/L) combined with DAP (20.6 mg/L) anti-biofilm formation was similar to DAP monotherapy (47.1 mg/L).

3.4 Antibacterial time–kill assays

Four VRE isolates (4942, 12022, 19372, 23760) with stronger ability of biofilm formations and ATCC 29212 were treated with antibiotics at average steady-state serum concentrations. The results were shown in Fig. 2. Among monotherapy time–kill studies, DAP showed bactericidal activity against four VRE isolates at 24 h. And the bactericidal activity of DAP was concentration-dependent against VRE. It is noteworthy that FM (83 mg/L) combined with DAP (20.6 mg/L) and DAP monotherapy (47.1 mg/L) reduced the population of four VRE isolates to zero without re-growth at 24h.

4. Discussion

E. faecium and *E. faecalis* are major nosocomial pathogens worldwide [18]. So far, the increasing prevalence of VRE is posing a constraint on therapeutic options. Furthermore, the effects of antibiotics for VRE were reported limit *in vitro* and *in vivo* studies. In the present study, VRE showed sporadic tendency. The resistance determinant of VAN in these strains was due to VanA-type cassette. Virulence genes in VREs were more than VREm. In addition, FM (83 mg/L) combined with DAP (20.6 mg/L) showed pronounced biofilm elimination effects and bactericidal activity.

Although it has been reported that 0.3–20% VRE were DAP resistance [19–20], all isolates in our study were sensitive to DAP. The eight isolates were all high-level VAN resistance. In terms of antibiotic resistance, one of the most relevant antibiotic resistance traits acquired by enterococci is resistance to vancomycin due to acquisition of the van gene clusters [21]. Furthermore, VREm frequently exhibits resistance to ampicillin and high-level resistance to aminoglycosides [22–23].

The development of VAN resistance is usually through the acquisition of *van* genes. Among the nine *van* genotypes reported so far, *vanA* (80%-90%) and *vanB* (10%-20%) are the most predominant [24]. The *vanA* operon usually consists of five genes (*vanHAXYZ*) for glycopeptide resistance, two regulatory genes (*vanRS*), a transposase (*orf1*)/resolvase (*orf2*) region, and is usually carried by a Tn3-type transposon [24–25]. Genetic variations, such as deletions and/or addition of some insertion sequences, have been reported in Tn1546 [26]. The VanA operon is carried on Tn1546-type transposons, which display a high degree of heterogeneity. Moreover, high recombination rates and the acquisition of mobile elements in the genome of E. faecium also have driven this evolutionary process [27]. In addition, the enrichment of virulence determinants, such as surface proteins and phosphotransferase systems (particularly PTSclin, a putative factor found to contribute to the intestinal colonization in a murine model) seems to provide an advantage to the hospital adaptive process [28]. Furthermore, functional gene groups, such as those involved in galactosamine metabolism, bile hydrolysis and phosphorus utilization,

are also abundant in *E. faecium* clinical strains compared to non-clinical isolates, suggesting that specific metabolic factors have also facilitated adaptation.

Several studies have investigated the importance of putative virulence genes in VRE, but there are no clear conclusions as to what constituents decisively contribute to the pathogenicity of VRE yet [29]. The only virulence genes confirmed to be associated with VRE infection are the enterococcal surface protein gene (*esp*) and the hyaluronidase gene (*hyl*) [30–31]. The putative virulence factors include proteins that attack several different constituents of cells, such as cytolysin that targets cell membranes, as well as gelatinase and serine protease that attack various proteins such as collagen, fibrinogen, and insulin. In addition, binding proteins such as collagen-binding protein, enterococcal surface protein, and aggregation substance are putative virulence factors [32]. We found virulence genes in *E. faecalis* were more than *E. faecium*. Furthermore, it has been suggested that the different factors may have different roles in the different resistant *Enterococcus* species.

It is of note that severe infection with VRE does require treatment, but due to the high antibiotic resistance, and the innate ability of enterococci to develop resistance toward new compounds quickly, there are few effective therapies available [33]. We found LNZ and DAP monotherapy showed effective anti-biofilm formation during 72 hours. In addition, DAP showed bactericidal activity against four VRE isolates at 24 h. Notably, one large study found that among 138 patients that received linezolid treatment, there was approximately 18% overall mortality [34]. When daptomycin is administered in higher doses, there is a concern for the toxicity of the drug, as it has been found to cause increased creatine kinase levels, resulting in muscle toxicity [35]. Interestingly, FM combined with LNZ or VAN showed better anti-biofilm effect than monotherapy. Furthermore, FM (83 mg/L) combined with DAP (20.6 mg/L) and DAP monotherapy (47.1 mg/L) reduced the population of four VRE isolates to zero without re-growth at 24h. Previous reports demonstrated FM combined with DAP had synergistic effects as well [17, 36]. Therefore, the combination of FM and DAP for patients with VRE infections, especially for patients with renal impairment, is of great significance for further clinical trials.

5. Conclusions

The high-level resistance determinant of sporadic VRE in the present study was due to VanA-type cassette. The most efficient regimen of bactericidal effect against VRE and biofilm inhabitation was the combination of FM and DAP. Further *in vivo* investigation and clinical trials are needed to define the effect of different drug combinations.

Declarations

Consent for Publication. All authors have seen and approved the content and fulfil the journal's requirements for authorship.

Availability of data and material. The BioProject for VRE has been deposited at GenBank under the accession PRJNA662846 and PRJNA662849.

Competing interests. None.

Funding. This work was supported by Department of Health of Zhejiang province [2019RC003].

Authors' contributions. WY and YQQ developed the concept and designed the experiments. WY, YHJ, and SBY isolated bacteria. WY, YHJ, and LZ performed the laboratory measurements. WY and XHJ analysed the data. YQQ gave conceptual advice. WY wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages.

Ethics approval. We declare no ethical competing interest. In our study, we did not perform any experiments with animals or higher invertebrates, neither performed experiments on humans nor the use of human tissue samples. Our data have been originated from bacteria, not linked to clinical information.

Acknowledgements. Not applicable.

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Figures

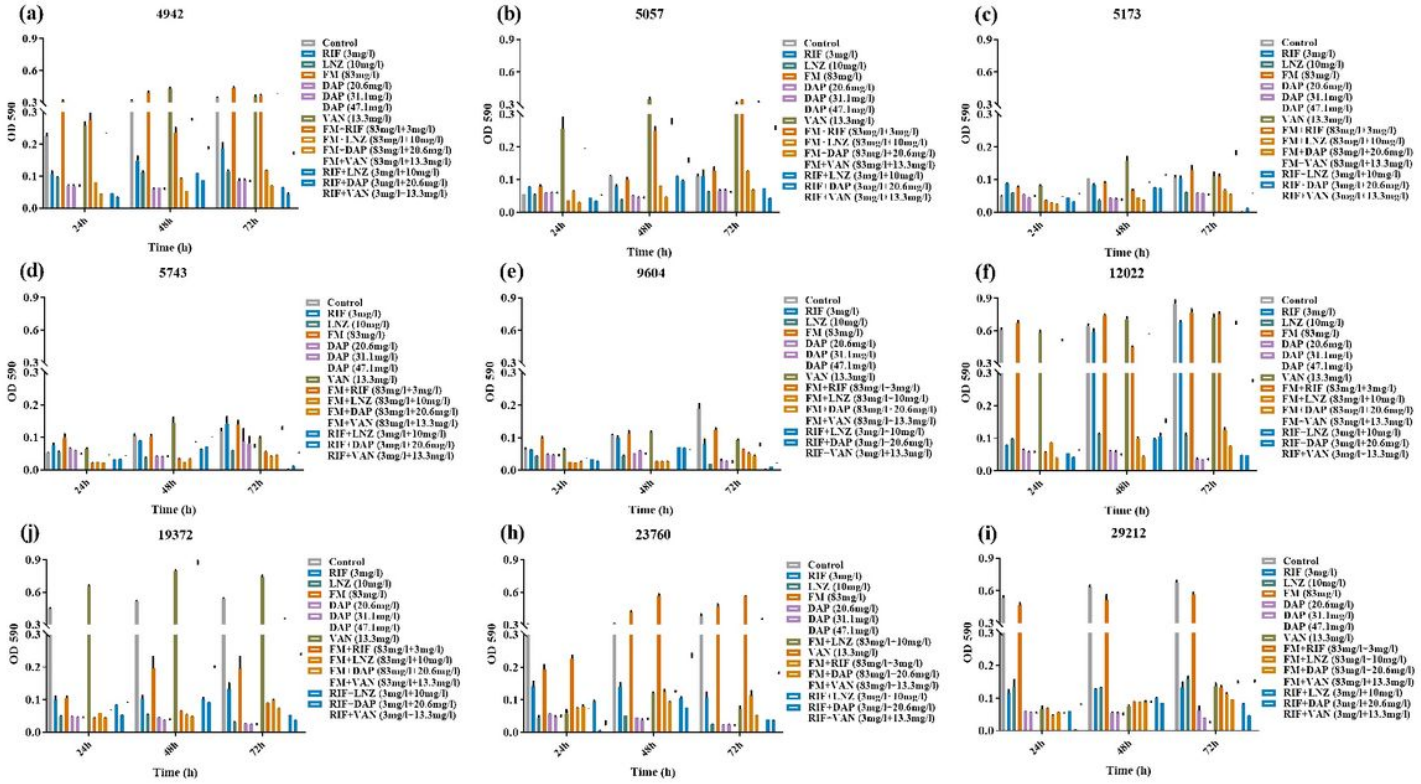

Figure 1

The anti-biofilm formation of rifampin (RIF), linezolid (LNZ), fosfomycin (FM), daptomycin (DAP) monotherapy and in combination against 8 VRE isolates and ATCC 29212 for 24 hours, 48 hours and 72 hours. (a) 4942; (b) 5057; (c) 5173; (d) 5743; (e) 9604; (f) 12022; (g) 19372; (h) 23760; (i) 29212.

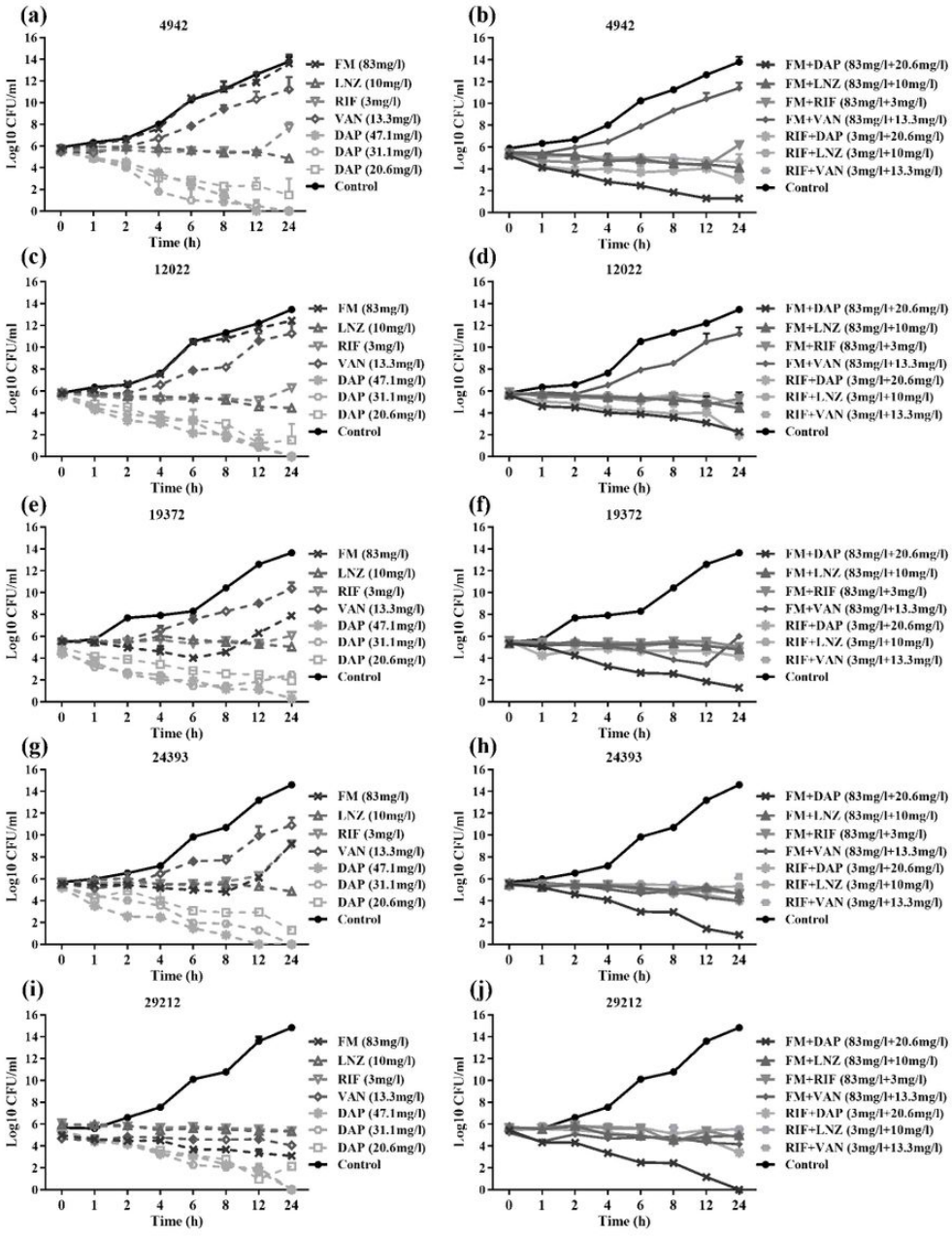

Figure 2

In vitro time–kill assays of fosfomycin (FM), rifampin (RIF), vancomycin (VAN), linezolid (LNZ), daptomycin (DAP) monotherapy and in combination against 4 VRE isolates and ATCC 29212. (a)(b) 4942; (c)(d) 12022; (e)(f) 19372; (g)(h) 23760; (i)(j) ATCC 29212. The dotted lines indicate monotherapy and the solid lines indicate combination therapy.

Supplementary Files

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