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ORIGINAL RESEARCH

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

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Purpose: 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 studies indicated FM (83 mg/L) combined with DAP (20.6 mg/L) and DAP monotherapy (47.1 mg/L) had bactericidal effect against VRE isolates at 24h.

Conclusion: High-level resistance determinant of VAN in tested isolates was due to VanA-type cassette. FM combined with DAP is a potential therapeutic option for VRE infections.

Keywords: vancomycin, daptomycin, combination therapy, biofilm

Introduction

Vancomycin resistant *Enterococci* (VRE) are increasingly becoming public health threat for hospitals worldwide. VRE infections caused significant mortality, ranging from 19% to 63%.[1,2] In previous years, most VRE infections were caused by *E. faecalis*.[3] However, since 2002 an increase in the prevalence of vancomycin-resistant *Enterococcus faecium* (VREm) has been observed, with reports of VREm being as common as vancomycin-resistant *E. faecalis* (VREs).[4] 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.[5]

The opportunistic invasive VRE infections are often broadly resistant to available antibiotics. Combination therapies, such as gentamicin and β-lactams, were

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reported to achieve reliable bactericidal effect for enterococcal endocarditis.[6] However, isolates with high-level resistance of gentamicin do not show synergism during combination. Current effective treatments for VRE included quinupristin/dalfopristin, teicoplanin, telavancin, linezolid (LNZ), and daptomycin (DAP).[7] 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 tigecycline of fosfomycin (FM), rifampin (RIF), vancomycin (VAN), LNZ, DAP alone and in combination against VRE.

Methods

Bacterial Strains and Antibiotic Susceptibility Test

A total of 8 VRE strains were isolated from clinical specimens for diagnosis and frozen at −80◦C in our laboratory. The pure cultures were put on identification plate and add 2 μL mixed liquid including trifluoroacetic acid, acetonitrile and distilled water. After drying, all isolates were definite identification 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.[8] 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 29,212 was used as quality control.

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).

Anti-Biofilm Testing

All VRE isolates and ATCC 29,212 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.[9] All experiments results were from three separate experiments.

Anti-Complement Killing Test

Mouse serum was purchased from Dalian Guangzhou Ruite Biotechnology (Guangzhou, China). 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:

$$\text{Bacterial survival rate} = (\text{number of colonies with normal serum}/\text{number of colonies with inactivated serum}) \times 100\%.$$

ATCC 29,212 served as the control strain.

Time–Kill Assays

The bactericidal activities of FM, VAN, and DAP alone or in combination against four VRE (4942, 12,022, 19,372, 23,760) and ATCC 29,212 were investigated using the time–kill method.[10] The antibiotic concentrations were calculated according to steady-state concentrations of drug in humans as described previously.[11] The following concentrations were used: FM 83 mg/L;[11] LNZ 10 mg/L;[12] RIF 3 mg/L;[13] VAN 13.3 mg/L;[14] DAP 20.6 mg/L, 31.1 mg/L and 47.1 mg/L;[15] 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. Each test had three replications.

Results

Antimicrobial Susceptibility and Multi-Locus Sequence Typing (MLST)

There were two VREs and six VREm isolates included in this study. Eight VRE isolates obtained from culture including urine (n = 3), bile (n = 1), and blood (n = 4) (Table 1). All isolates showed high-level resistance to VAN, whereas they were susceptible to tigecycline, LNZ, DPA (Table 1). Only two E. faecalis isolates (4942 and 12,022) were susceptible to RIF.

MLST revealed two different STs for VREs isolates 4942 (ST4) and 12,022 (ST179). There were four STs (ST412, ST564, ST78, ST17) for VREm.

Resistance Genes and Virulence Genes

Isolate 23,760 has the least number of resistance genes. Three genes *vanRA, vanSA, vanYA* were found in all isolates. VanA, VanXA were not found in 12,022 and 23,760. In addition, *vanHA* was not identified in 12,022 and *vanZA* was not found in 23,760 (Supplemental Table 2). The *vanA* operon was carried on Tn1546 transposon in six isolates (4942, 5057, 5173, 5734, 9604, 12,022). Isolates 4942, 5734, and 9604 had genomic island including *vanZ, vanY, vanX, vanA, vanH, vanS, vanR* (Supplemental Figure 1), while 5057, 5173, and 12,022 had genetic rearrangements in Tn1546 transposon.

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.

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 Figure 2).

As shown in Figure 1 and Supplemental Table 4, the biofilm formations of isolate 5057, 5173, 5743, 9604 were less than other four VRE isolates. Except for isolate

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*)	12,022 (*E. faecalis*)	19,372 (*E. faecium*)	23,760 (*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

Abbreviations: 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.

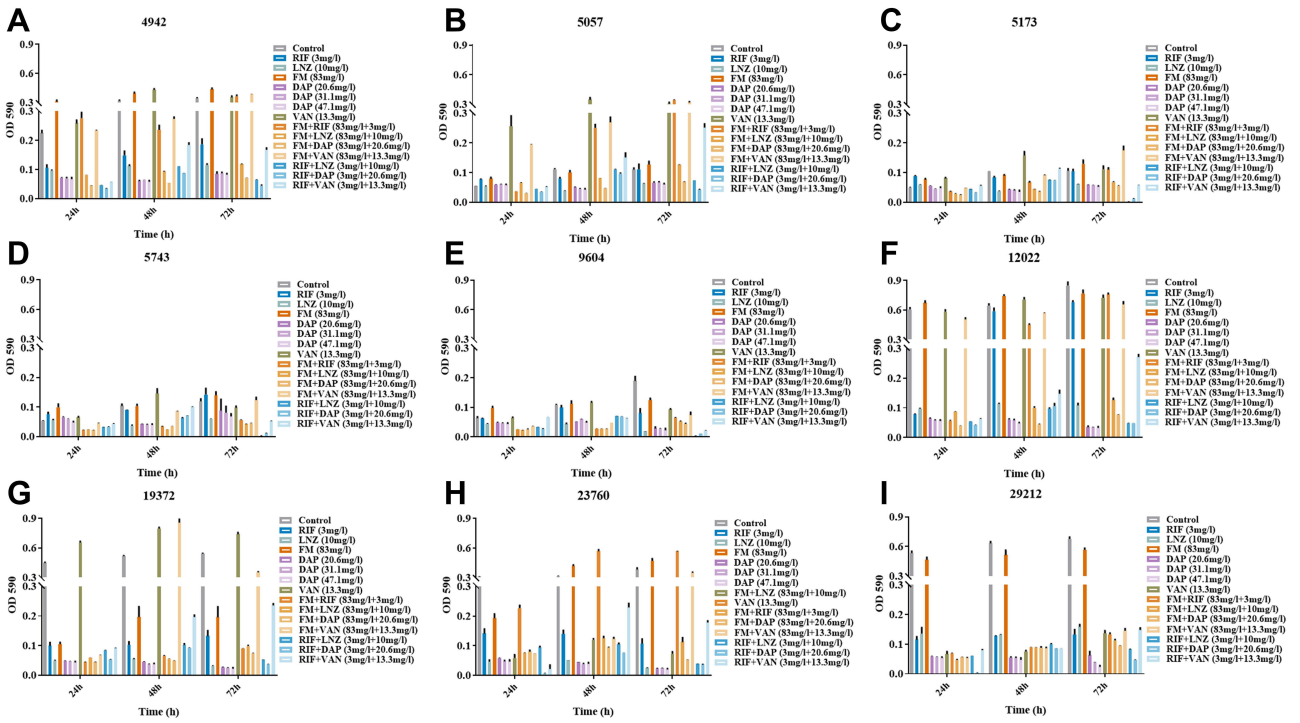

Figure 1 The anti-biofilm formation of rifampin (RIF), linezolid (LNZ), fosfomycin (FM), daptomycin (DAP) monotherapy and in combination against 8 VRE isolates and ATCC 29,212 for 24 hours, 48 hours and 72 hours. (**A**) 4942; (**B**) 5057; (**C**) 5173; (**D**) 5743; (**E**) 9604; (**F**) 12,022; (**G**) 19,372; (**H**) 23,760; (**I**) ATCC 29,212.

19,372, 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).

Time–Kill Assays

Four VRE isolates (4942, 12,022, 19,372, 23,760) with stronger ability of biofilm formations and ATCC 29,212 were treated with antibiotics at average steady-state serum concentrations. The results were shown in Figure 2. Among monotherapy time–kill studies, DAP showed bactericidal activity against four VRE isolates at 24 h. The bactericidal activity of DAP was concentration-dependent. 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 regrowth at 24h.

Discussion

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, 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.

Eight isolates showed high-level resistance to VAN, relating to VanA-type cassette. So far, one of the most relevant VAN resistance traits is the acquisition of *van* genes.[16] *vanA* (80–90%) and *vanB* (10–20%) are the most predominant among 9 *van* genotypes.[17] The *vanA* operon usually consists of five genes (*vanHAXYZ*) for glycopeptide resistance, two regulatory genes (*vanRS*), a transposase (*orf1*)/resolvase (*orf2*) region.[17] Tn1546 transposon was contributed to the increase of VRE infections.[18] Genetic variations, such as deletions and/or addition of some insertion sequences, have been reported in Tn1546.[19] Consistent with previous studies, three VRE isolates (5057, 5173, and 12,022) were found polymorphisms in upstream of *vanR* among Tn1546 as well.[20] Due to horizontal transfer of plasmids, it is necessary to reinforce managements to prevent spreading of VRE.

Several studies have investigated the importance of putative virulence genes in VRE, however, there are no

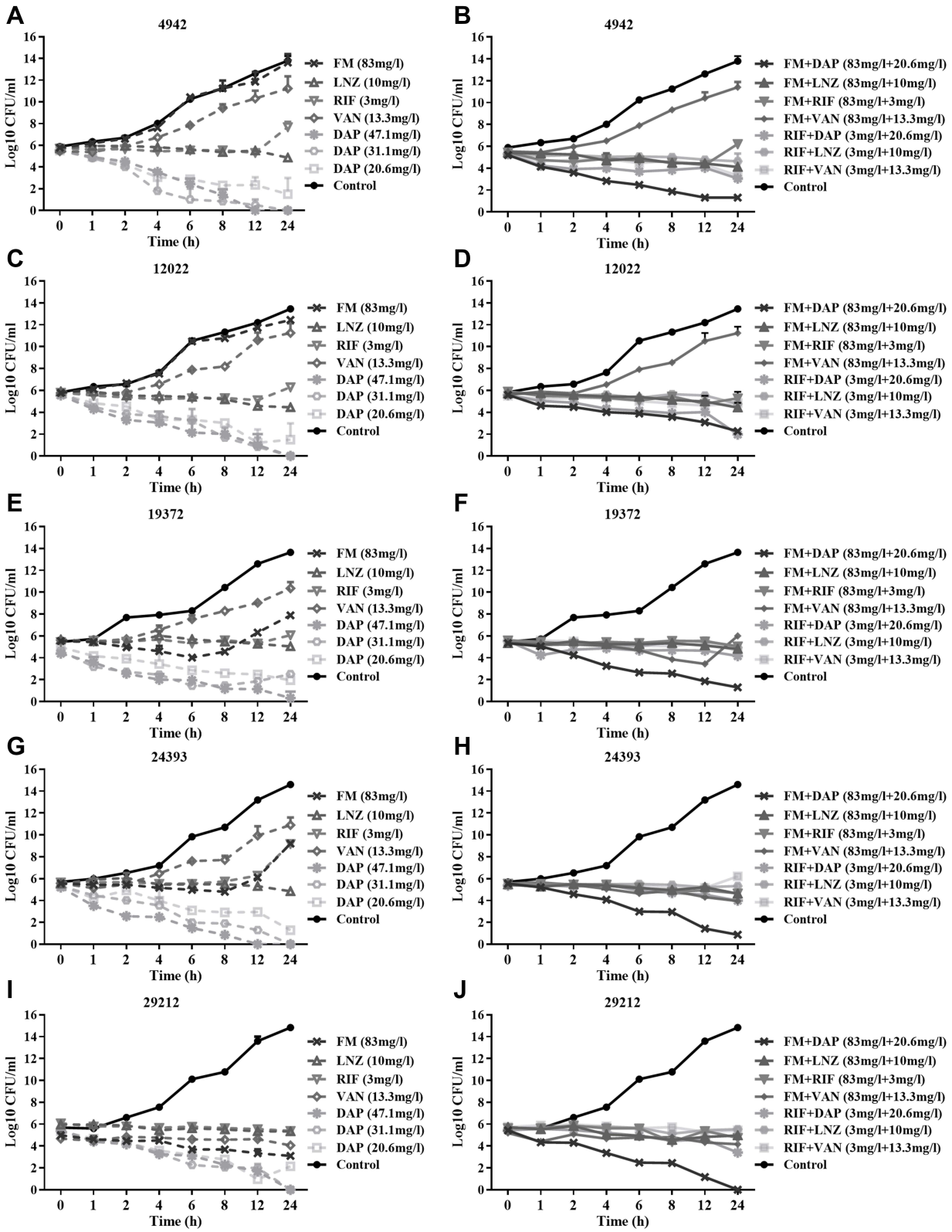

Figure 2 In vitro time–kill assays of fosfomycin (FM), rifampin (RIF), vancomycin (VAN), linezolid (LNZ), daptomycin (DAP) monotherapy and in combination against 4 VRE isolates and ATCC 29,212. (**A** and **B**) 4942; (**C** and **D**) 12,022; (**E** and **F**) 19,372; (**G** and **H**) 23,760; (**I** and **J**) ATCC 29,212. The dotted lines indicate monotherapy and the solid lines indicate combination therapy.

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clear conclusions as to what constituents decisively contribute to the pathogenicity of VRE yet.[21] We found virulence genes in *E. faecalis* were more than *E. faecium*. The only virulence genes confirmed to be associated with VRE infection are the enterococcal surface protein gene (*esp*) and the hyaluronidase gene (*hyl*).[22,23] 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.[24]

Notably, a few effective therapies are available for VRE infections.[25] Other options, such as pump inhibitors and essential oils, have been proved to have antibacterial activity against multidrug-resistant *Enterococci* in vitro.[26,27] Although previous studies showed 0.3% to 20% VRE were resistance to DAP, all isolates in our study were sensitive to DAP.[28,29] LNZ and DAP monotherapy showed effective anti-biofilm formation during 72 hours. It is of note that LNZ treatment for VRE bloodstream infection was associated with higher mortality and microbiologic failure in comparison to DAP.[30] DAP showed bactericidal activity against four VRE isolates at 24 h in the time-kill assay. There is a concern for the toxicity of higher doses of DAP due to increase of creatine kinase levels and muscle toxicity.[31] Fortunately, similar to DAP monotherapy (47.1 mg/L), FM (83 mg/L) combined with DAP (20.6 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.[15,32] 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.

In conclusion, 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.

Data Sharing Statement

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

Ethics Approval

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 which were frozen for antimicrobial resistance monitoring, not linked to clinical information. Therefore, our research was exempt from ethics approval.

Consent for Publication

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

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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