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ORIGINAL ARTICLE

Efficacy of Vancomycin with Fosfomycin against Vancomycin-Resistant Enterococcus Strains Using Agar Dilution Method

Gulseren Aktas

Istanbul University, Istanbul Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkey

SUMMARY

Background: ***Enterococcus faecalis*** **and** ***Enterococcus faecium*** **can cause community-acquired and nosocomial infections. Combination antibiotic therapies have a distinct advantage over monotherapies in terms of their synergistic effect. In the study, it was aimed to investigate** ***in vitro*** **activity of vancomycin combined with fosfomycin by agar dilution method against VRE strains.**

Methods: **A total of 30 clinical VRE strains were included in the study. Bacterial identifications of the strains were undertaken using conventional routine methods. The resistance to vancomycin was investigated using the broth microdilution method compared to fosfomycin by agar dilution method, and the results were interpreted in accordance with the CLSI guidelines. All experiments contained 25 mg/mL glucose-6-phosphate for fosfomycin. The fractional inhibitory concentration (FIC) indexes (FICI) were interpreted as synergism, FICI ≤ 0.5. Additionally, two strains in 30 VRE were studied to determine the time-kill curves to verify the synergistic results. Both antibiotics were studied at 1 x MIC in the tests. Viable counts were determined at 0, 4, 8, 12, and 24-hour intervals. Time-kill curves were constructed by plotting mean colony forming unit counts versus time.**

Results: **Susceptibility rate to fosfomycin was found at 16.6% (5/30). The $MIC_{50,90}$ and MIC_{range} values of antimicrobials were 512, 512, and 512 - 1,024 mg/L for vancomycin, and 128, 128, and 64 - 160 mg/L for fosfomycin. The rate of synergism was found as 100%.**

Conclusions: **The result shows that the combination of vancomycin with fosfomycin gives hope that it may be an option in the treatment of infections caused by VRE.**

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Correspondence:
Gulseren Aktas
Millet Street
Istanbul Faculty of Medicine
Department of Medical Microbiology
34093 İstanbul
Turkey
Phone: +90 212 414 2000/32417
Mobile: +90 555 379 4557
Fax: +90 212 414 2037
Email: gulserena2001@yahoo.co.uk

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KEY WORDS

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INTRODUCTION

Vancomycin-resistant enterococci (VRE) started to emerge in European countries and then in the United States of America [1] after it was first reported in 1988 [2]. The VRE strains then became important factors of hospital infections which mainly cause epidemics particularly in dialysis, transplantation, hematology, and intensive care units [3,4]. The increase of strains with multiple antibiotic resistance makes the treatment more

difficult in addition to the frequent isolation of enterococci as the infectious agent [4,5].
Vancomycin-resistant enterococci associated infections have been increasing in recent years despite the emergence of antimicrobial materials in the treatment of pathogenic bacteria with multiple antibiotic resistance, and the number of antimicrobial agents to be used in treatment is limited. Another option that can be used in the treatment of the infections of resistant bacteria is the use of a combination of the antimicrobial substances. Combination treatments increase the possibility of treatment owing to their synergistic effect, if there is any, and wide effect spectrum. In addition, the rapid increase in the rates of drug resistance in all bacteria worldwide and the inadequacy in the development of new antibiotics necessitated the formation and development of combined use of antibiotics studies [6,7].
Fosfomycin is phosphonic acid derivative that exhibits bactericidal antimicrobial activity by interfering with cell wall synthesis in both Gram positive and Gram negative bacteria. It represents its own class of antibiotics and no other member of this class is currently approved worldwide. It inhibits the synthesis of peptidoglycan in an early-stage by blocking the formation of N-acetylmuramic acid. This mechanism of action of fosfomycin makes the probable cross-resistance to other antibiotics (almost) impossible. Fosfomycin provides the possibility of the combined use with many antimicrobials [8,9]. In addition, fosfomycin is an antibiotic worth investigation because it is an antibiotic with single dose superiority owing to its wide effect spectrum, low adverse toxic effects, and longer half-life [10].
The aim of the present study was to investigate a new alternative to expand treatment options of infections caused by vancomycin-resistant enterococcal strains.

MATERIALS AND METHODS

Bacterial Isolates and Identification

A total of 30 clinical VRE strains were studied. Out of the 30 strains, 14 were isolated from blood samples between 2007 and 2014, and 16 were isolated from urine between 2013 and 2016 from different patients who were admitted to different clinics of the university hospital. These VRE strains were obtained using conventional methods. They were identified as *Enterococcus* spp. if they had the following properties: Gram-positive; catalase negative; ability to grow in 6.5% sodium chloride and 40% bile, and hydrolyzed esculin; and positive results in pyrrolydonyl arylamidase tests (PYR; BD; USA).
The species were identified using biochemical and physiological tests such as arginine dihydrolase, hipporate hydrolysis, growth in pyruvate, pigment production, motility, and carbohydrate utilization tests [11]. To verify the results, intrinsic antimicrobial resistance profiles were investigated, and all strains were tested for susceptibilities to ampicillin (10 μg: Oxoid, UK), imipenem (10 μg: BBL™, USA), and quinupristin/dalfopristin (Q/D) (15 μg: Oxoid, UK). Nitrocefin discs (BD BBL™, Cefinase, USA) were used to investigate the production of β-lactamase enzyme [12].

Antimicrobial agents

The powder of vancomycin (Multicell, USA) and fosfomycin (Bilim Ilaç A.Ş., Istanbul) antimicrobials were used in the broth microdilution, agar dilution, checkerboard, and time-kill tests. The vancomycin resistance of the strains was investigated by disc diffusion tests (30 μg: Oxoid, England), and then, verified by the broth microdilution test to classify them as VRE. Teicoplanin disks (Glentham Life Sciences Ltd, UK) were also used for the phenotyping of the VRE strains.

Minimum inhibitory concentration (MIC) testing

The susceptibility of the strains to the agents was investigated using the broth microdilution method for vancomycin and the agar dilution method for fosfomycin described by the Clinical and Laboratory Standards Institute (CLSI) [13,14]. The agents used were prepared in accordance with the guidelines of the CLSI and the proposals of the manufacturing firms.

Broth microdilution method

The broth microdilution tests for vancomycin were conducted using cation-adjusted Mueller-Hinton II broth (CAMHB) (BBL™, Becton, Dickinson and Company, USA). The inoculum of each strain for the test was adjusted to achieve a final inoculum of 10^5 - 10^6 cfu/mL in the wells of microplates. The MIC was defined as the lowest concentration of vancomycin that resulted in the complete inhibition of visible growth after 24 hours of incubation at 35°C.

Agar dilution method

The agar dilution tests for fosfomycin were conducted using Mueller-Hinton II agar (BBL™, Becton, Dickinson and Company, USA) supplemented with 25 mg/L of glucose-6-phosphate (Sigma Aldrich Chemie GmbH, Germany). The inoculum of each strain on the agar plate after inoculation was 10^4 cfu in each drop of 2 μL. The MIC was defined as the lowest concentration of fosfomycin not seeing any growth on agar surface after overnight incubation at 35°C. Both results of the tests were interpreted in accordance with the CLSI guidelines. Additionally, after one strain (MIC ≥ 256 mg/L) was found as resistant in the first evaluation, the agar dilution method was studied in the internal concentrations of fosfomycin between 128 and 256 mg/L, as 160, 192 and 224 mg/L. The quality-control testing procedures in the both tests were performed by using *Staphylococcus aureus* ATCC 29213 and/or *Enterococcus faecalis* ATCC 29212 as reference strains in each run.

Broth microcheckerboard dilution testing

The *in vitro* activities of the antibiotics in combination were assessed using the broth microcheckerboard meth-

od [15]. The concentrations of antibiotics in combinations were based on two dilutions above the MICs and four dilutions below. The dilutions of antibiotics were performed in microtiter plates by preparing serial dilutions of one antibiotic horizontally and another vertically. The final inoculum of strains was approximately 10^5 - 10^6 cfu/mL in the microplate wells. After 24 hours of incubation at 35°C, the MIC values of each antibiotic alone in combination were noted as the lowest concentration of antibiotic combination that resulted in no visible growth. The fractional inhibitory concentration (FIC) indexes (FICI) were calculated for each combination using the equation, $FIC_A + FIC_B = FICI$, where FIC_A is the MIC of drug A in combination divided by the MIC of drug A alone, and FIC_B is the MIC of drug B in combination divided by the MIC of drug B alone. Synergism was indicated by FICI <= 0.5 [15].

Time-kill testing

Two strains which were randomly selected from the 30 VRE were studied to determine the time-kill curves to verify the obtained synergistic results of the checkerboard method. For each strain, antibiotics were studied alone and in combination at the 1 x MIC concentration. An antibiotic-free control was included as growth control. An inoculum was produced by diluting the culture in CAMHB, which was obtained by overnight culture of the strain incubated in a calibrated shaking water bath at 70 cycles/minute at 35°C (GFL - 1092, Kisker Biotech GmbH and Co., KG, Germany). The inoculum was added to all flasks to yield a final concentration of approximately 1 x 10^6 cfu/mL [7]. The flasks had a final volume of 10 mL, and the incubation was continued under the same conditions. The viable counts were determined at intervals of 0, 4, 8, 12, and 24 hours after inoculation by subculturing 0.1 mL from each repetitive serial dilution to 10^{-7} in Eppendorf tubes containing sterile saline (0.9% NaCl). The subcultures were done on plates containing Tryptic Soy Agar (TSA) (Difco™, Becton, Dickinson and Company, France) and were incubated at 35°C for 24 hours.

Time-kill curves were constructed by plotting the mean colony forming unit (cfu) counts versus time. The synergy of the combination was defined as a reduction in colony count of ≥ 2 $\log_{10}$ cfu/mL or more at 24 hours with the combination compared with the most active single agent alone [7].

RESULTS

Of the 30 clinical VRE strains isolated, there were 28 identified as *Enterococcus faecium* and 2 identified as *Enterococcus faecalis* based on the tests results. The 28 *E. faecium* strains were also found to be resistant to ampicillin and imipenem and susceptible to Q/D, while the 2 *E. faecalis* strains had the opposite results. None of the strains produced β-lactamase enzyme. All VRE strains were resistant to vancomycin and teicoplanin according to both disk diffusion and broth microdilution tests. The $MIC_{50,90}$ and MIC_{range} values were 512, 512, and 512 - 1,024 for vancomycin and 64, 128, and 16 - 128 mg/L for teicoplanin. The results showed that all strains had VanA glycopeptide resistance phenotype. The $MIC_{50,90,range}$ values of fosfomycin were found as 128, 128, and 64 - 160 mg/L, respectively. One strain which was found as resistant (MIC; 250 mg/L) to fosfomycin in the first evaluation, was then found as MIC: 160 mg/L. As a result, five of the strains were evaluated as susceptible to fosfomycin (MIC; $\leq$ 64 mg/L), 25 (83.3%) were less sensitive and no resistant strain was detected (Table 1). The rate of the synergistic effect of the vancomycin-fosfomycin combination for the 30 VRE strains was found to be 100% (30/30) in the broth microcheckerboard method (Table 2). The distribution of FICI values of the combination were also shown in the same table. The MIC_{50}, MIC_{90} and MIC_{range} values of each antibiotic alone in the combination were found as 32, 32, and 16 - 32 for vancomycin, and 16, 32, and 8 - 32 mg/L for fosfomycin, respectively (Table 3). All strains were found resistant to vancomycin and sensitive to fosfomycin in accordance with these concentration values. The two randomly selected VR-*E. faecium* strains were also studied using the time-kill method to confirm the results. The comparative results of both checkerboard and time-kill studies results were shown in Table 4. The combinations studied were found to be synergistic at the 1 x MIC concentrations of both antibiotics against all 2 *E. faecium* strains (Figure 1, 2). Synergy was defined at 24 hours in strain 1 (Figure 1) and 12 hours in strain 2 (Figure 2). No bactericidal effect of the combination was detected in two VRE strains.

DISCUSSION

The continuously increasing problem of antibacterial resistance has been seen frequently in the world, and it has threats for potential consequences. Another option that can be used in the treatment of the infections of resistant bacteria is the use of a combination of the antimicrobial substances [7]. Fosfomycin was initially described and isolated in 1969 from cultures of *Streptomyces* species [16]. Today, it is produced synthetically. Fosfomycin has broad-spectrum activity against various Gram-positive and Gram-negative bacteria by irreversibly blocking bacterial cell wall synthesis at an earlier stage than beta-lactam or glycopeptide antibiotics. Vancomycin acts by preventing cell wall production by binding to the terminal D-alanyl-D-alanine dipeptide (D-Ala-D-Ala) of the pentapeptide chain. Nonspecific binding of glycopeptides to the cell wall also occurs, and this nonspecific binding appears to affect the action of autolysins involved in the physiological hydrolysis of peptidoglycan [12]. Although the mechanism of interaction between antimicrobials is unclear, it may be possible that this combination may increase the nonspecific

Table 1. The MIC values of antimicrobial agents and susceptibility rates.

Agent	MIC values (mg/L)			Susceptibility* n (%)
	MIC_{50}	MIC_{90}	MIC_{range}	
Vancomycin (VAN)	512	512	512 - 1024	0
Fosfomycin (FOS)	128	128	64 - 160	5 (16.7)

* - ***Susceptibility breakpoints*: VAN ≤ 4, FOS ≤ 64, mg/L [14].**

Table 2. The distribution of FICI values of the combination against 30 VRE strains.

Combination	Distribution of FICI values (n)					Synergism n (%)
	0.1	0.2	0.3	0.4	> 0.5	
VAN + FOS	8	16	5	1	-	30 (100)

Table 3. The MIC values and susceptibility rates of each antimicrobial alone in combination using checkerboard test.

VRE (n = 30)	MIC values (mg/L)			Susceptibility (%)
	MIC_{50}	MIC_{90}	MIC_{range}	
Vancomycin	32	32	16 - 32	0
Fosfomycin	16	32	8 - 32	100

Table 4. The comparative results of dilution, checkerboard, and time-kill studies against the two VRE strains.

VRE No.	MIC results (mg/L)			FICI	Time-Kill Results*
	Dilution		Checkerboard*		Synergistic Effect**
	MIC_{VAN}	MIC_{FOS}	$MIC_{VAN/FOS}$		
1	512	128	32/16	0.2	+
2	512	160	32/16	0.2	+

* **- Concentrations of both antimicrobials were at 1 x MIC for each strain in the tests,** ** **- ≥ 2 log_{10} CFU/mL reduction.**

binding of vancomycin to the bacterial cell wall and causes peptidoglycan hydrolysis. It may also be thought that glucose-6-phosphate may lead to more vancomycin molecules to be bound and, then, may cause much more effective action of vancomycin [8].

It is possible that fosfomycin may not be as effective today as it was in the past. In most infections, fosfomycin is given in combination with another antibiotic. Unfortunately, data on the efficacy of fosfomycin in combination with newly developed glycopeptide antibiotics such as oritavancin, telavancin or dalbavancin are currently not available in scientific literature.

In a study, vancomycin and fosfomycin in combination against *Staphylococcus aureus* and *S. epidermidis* strains recorded that the results were found as synergistic, additive and indifferent, whereas in combination with teicoplanin and fosfomycin were found as synergistic against *E. faecalis* and *S. aureus* [8].

Fosfomycin has historically shown excellent *in vitro* activity against vancomycin-resistant enterococci, and therefore might be considered as a treatment option for infection caused by this organism. However, informa-

Figure 1

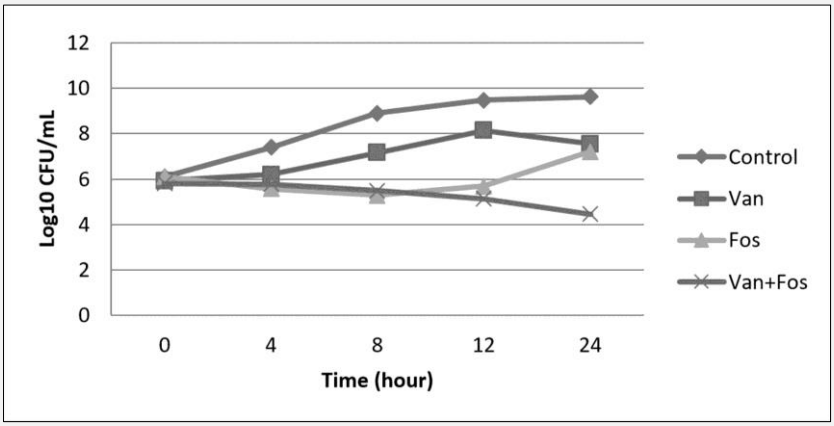

Figure 2

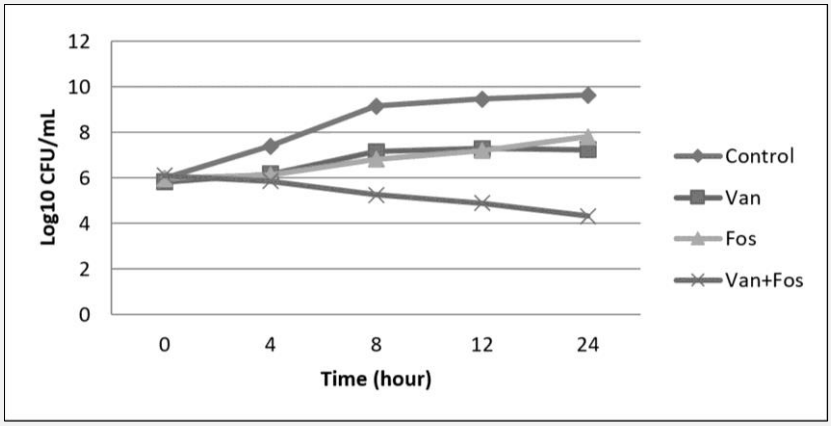

Figure 1, 2. Time-kill curves obtained with combinations of vancomycin and fosfomycin against two VRE strains.

tion regarding the activity of fosfomycin against VRE in the setting of increasing fosfomycin use is limited [17-20]. Some studies reported that fosfomycin susceptibilities for VR-*E. faecium* were ranging from 30% - 100% [21,22]. Another study evaluated *in vitro* activity of fosfomycin against 75 urinary tract isolates of vancomycin-resistant enterococci, and 51 out of 52 *E. faecium* and all *E. faecalis* isolates tested were susceptible or intermediate to fosfomycin [23].

On the other hand, there are studies that were found resistant to fosfomycin or the sensitivity for fosfomycin was 30% in VR-*E. faecium* and 44% in VR-*E. faecalis* strains [17,24]. In our study, five VRE strains were susceptible and 25 were less susceptible to fosfomycin. No resistant strain was found, but most of them were less susceptible to fosfomycin.

Owing to the increase of drug resistance in pathogenic microorganisms, and the inadequacy in the development of new antibiotics, there has been an increase in the use of combined treatment of fosfomycin for infections of multiple resistant bacteria [6,8,25,26].

In this study, the combination of vancomycin and fosfomycin was found effective synergistically against all VRE strains for both fosfomycin sensitive (MIC $\leq$ 64 mg/L) and less sensitive (MIC > 64 - < 256 mg/L) VRE strains, and both *E. faecium* and *E. faecalis* strains. When the antibiotics tested were combined, the investigation of MIC values of each antibiotic alone and in combination was detected to be the concentration levels that could reached in the human body [10,18]. There is very limited experience for *in vitro* activity of fosfomycin in combination with vancomycin against VRE strains [24].

In the study, the antimicrobial efficacy increased in the combined use of fosfomycin and vancomycin. This combination may enhance the choices in treatment of infections caused by VRE strains which have a limited number of alternative treatment options after more *in vitro* and *in vivo* studies are conducted. Additionally, this synergistically effective combination of fosfomycin

may encourage the study of new synergistic combinations of fosfomycin with other antibiotics because of high drug resistance and very limited new antibiotics being discovered.

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Declaration of Interest:
The author declares that the research was conducted in the absence of any commercial or financial relationship, that could be construed as a potential conflict of interest.

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