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In vitro efficacy of fosfomycin-based combinations against clinical vancomycin-resistant *Enterococcus* isolates☆,☆☆,★

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ABSTRACT

Vancomycin-resistant (VR) enterococci (VRE) are increasingly important nosocomial pathogens, commonly causing catheter-related urinary tract infections or vascular catheter-related bloodstream infections. In this study, 10 *Enterococcus faecium* and 9 *Enterococcus faecalis* different pulsed-field gel electrophoresis genome-type VR clinical isolates were detected. The potential role of fosfomycin-based combination regimens for biofilm-related VRE infection is *in vitro* evaluated. Anti-VRE activities of fosfomycin, ampicillin, linezolid, minocycline, rifampicin, tigecycline, teicoplanin, vancomycin alone, or fosfomycin-based combinations were studied by time-kill method and a biofilm model. Of the fosfomycin-based combinations, a synergistic effect was particularly noted for teicoplanin against 89% of the VR *E. faecalis* isolates. In a biofilm model, only linezolid alone was able to reduce the bacterial loads, and the use of fosfomycin-based combinations, excluding rifampicin (40%), failed to enhance antibacterial activity against VR *E. faecium*. For *E. faecalis*, an inhibitory effect was evident using ampicillin alone or fosfomycin plus rifampicin (100%), tigecycline (56%), or teicoplanin (44%). However, an antagonistic effect was found for ampicillin plus fosfomycin against 2 of 3 of the VR *E. faecalis* isolates. The antibacterial activities of the drugs tested against VRE *in vitro* varied by species. Ampicillin exhibited potential activity against planktonic- and biofilm-embedded VR *E. faecalis*. Fosfomycin-based combinations may have enhanced antibacterial effects against VRE even in the biofilm model, and this observation warrants further clinical studies.

Enterococcus caused bloodstream, urinary tract, intra-abdominal, and surgical sites infections. Among them, especially Foley catheter–related urinary tract infection and venous catheter-related bloodstream infection (CRBSI) were often associated with the biofilm, in which the embedded organisms are more resistant to antibiotics than planktonically growing ones, indicative of the significant impact of biofilm formation (Mohamed and Huang, 2007). Accordingly, monotherapy with either vancomycin or ampicillin often fails to eradicate the biofilm-embedded enterococci in the patients with CRBSI, if the colonized catheter is left in situ (Sandoe et al., 2002). Fosfomycin has been considered to be an agent useful for therapy of uncomplicated lower urinary tract infections (UTIs) (Popovic et al., 2010). Given the excellent in vitro antibacterial activity of fosfomycin against vancomycin-resistant (VR) enterococci (VRE), good tolerability, and high urinary concentrations, fosfomycin has been suggested for the treatment of lower UTI due to VRE (Perri et al., 2002).Therefore, we study the inhibitory activity of these antimicrobial agents alone or in fosfomycin-based combinations against planktonic- and biofilm-embedded VRE isolates.

Eighty-three enterococci isolates from different body sites, including blood, wounds/abscess, joint fluid, urine, and other initially sterile specimens, were randomly obtained from the clinical microbiology laboratory of Chi-Mei Foundational Hospital and National Cheng Kung University Hospital, Tainan, Taiwan. Fifty-three were VRE, and 45 (85%) including 21 *Enterococcus faecalis* isolates and 19 *Enterococcus faecium* exhibited the biofilm-formation phenotype (O'Neill et al., 2007). Pulsed-field gel electrophoresis (PFGE) was performed among randomly selected 36 isolates, and 19 genetically similarity less than 80% VRE

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Table 1
The $MIC_{50/90}$ and MBEC of 8 antibiotics against 10 VR *E. faecium* and 9 VR *E. faecalis* isolates.

Drugs	MIC(μg/mL)			*E. faecium* (n = 10)			*E. faecalis* (n = 9)		
	S	I	R	$MIC_{50/90}$	Susceptible %	MBEC	$MIC_{50/90}$	Susceptible %	MBEC
Vancomycin	≤4	8-16	≥32	>128/>128	0	>1024	>128/>128	0	>1024
Ampicillin	≤8	≥16		>128/>128	10	>1024	1/2	100	>1024
Teicoplanin	≤8	16	≥32	16/32	40	>256	8/16	89	>256
Tigecycline	≤0.5			0.125/0.25	100	>16	0.25/0.25	100	>16
Linezolid	≤2	4	≥8	4/4	20	>1024	2/2	100	>1024
Rifampin	≤1	2	≥4	16/32	10	>1024	<0.25/1	89	>1024
Fosfomycin	≤64	128	≥256	128/128	30	>4096	128/128	44	>4096
Minocycline	≤4	8	≥16	16/32	20	>256	32/32	11	>256

isolates were selected for further time-kill and biofilm studies (Tenover et al., 1995). The *in vitro* susceptibility results of the 8 antibiotics against planktonic or biofilm VRE isolates are shown in Table 1. Minimal inhibitory concentrations (MICs) were determined by the agar dilution method according to the CLSI recommendations (CLSI, 2012), and minimum biofilm eradication concentration (MBEC) assay has been previously described (Tang et al., 2012).

All of the 19 isolates were susceptible to tigecycline. Lower susceptibility rates, including those for ampicillin and rifampin (10%), linezolid (20%), and teicoplanin (40%), were found against *E. faecalis*. However, for both VRE species, low susceptibility rates were observed with fosfomycin (30–44%) and minocycline (20–11%). The optical density values of the 10 VR *E. faecium* isolates and the 9 VR *E. faecalis* isolates, ranging from 0.36 to 3.0 (mean: 1.22) and 0.25 to 0.71 (mean 0.39), respectively, represent the ability of the isolates to form biofilms.

Time-kill experiments followed the methodology as defined by the CLSI (NCCLS, 1999); bacterial suspensions were diluted to the inoculum of 8.0×10^5 CFU/mL in 25 mL of fresh Mueller-Hinton broth with susceptible breakpoint drug concentrations of selected antibiotics due to the fact that susceptible breakpoint concentration is the concentration that is easily achieved in serum level.

Upon incubation of the 10 *E. faecium* isolates with fosfomycin, ampicillin, minocycline, rifampicin, teicoplanin, or vancomycin for 24 h, bacterial growth was not inhibited compared to the drug-free control (Fig. 1A). These results are compatible with the high resistant rates of *E. faecium* to the 6 drugs (Table 1). When the VR *E. faecium* isolates were co-cultivated with linezolid or tigecycline alone, the bacterial growth was mildly inhibited, with a less than 10-fold reduction compared to the initial inoculum. When fosfomycin was combined with linezolid or tigecycline, the antibacterial activity was enhanced, and the colony count decreased to 10^3 CFU/mL at 24 h (Fig. 1A). However, the synergistic effect of fosfomycin combined with ampicillin, tigecycline, teicoplanin, and vancomycin, in accordance with the defined as an at least 100-fold decrease in bacterial load between the combination and the most active constituent of 2 combined drugs after 24 h, was found in 30% of the 10 *E. faecium* isolates, and the effect of fosfomycin-minocycline was found in 40% of the isolates (Table 2).

Similar antibacterial activities of monotherapy (except ampicillin) and fosfomycin-based combinations (except fosfomycin plus rifampin or teicoplanin) were found against the 9 *E. faecalis* isolates (Fig. 1B). The inhibitory effect of ampicillin was the most potent, and the colony count decreased to less than 10^2 CFU/mL at 24 h (Fig. 1B). The synergistic antibacterial activity of fosfomycin in combination with teicoplanin was the most commonly seen and was found in 89% of the *E. faecalis* isolates (Table 2).

Susceptible breakpoint drug concentrations were also used to test inhibitory effects in the biofilm studies (Fig. 2A). Biofilm formation was modified from reference (O'Neill et al., 2007). In brief, the biofilm of individual isolates was prepared in 24-well culture plates. The medium in the well was removed by aspiration, and the biofilm was treated with antibiotics alone or fosfomycin-based combinations. The drug-containing medium was gently aspirated after one day at 37 °C, and the biofilm on the wells was incubated with fresh drug dilution for 5 consecutive days. The well containing the biofilm after 5-day incubation with antibiotics was sonicated by a water-table sonicator for 5 min. The disrupted biofilm was serially diluted and plated for viable cell counting at 37 °C with overnight culture. The detection limit of plating count is 100 CFU/mL. Serially diluted samples were plated onto the LB agar (Difco Laboratories, Sparks, MD, USA) plates

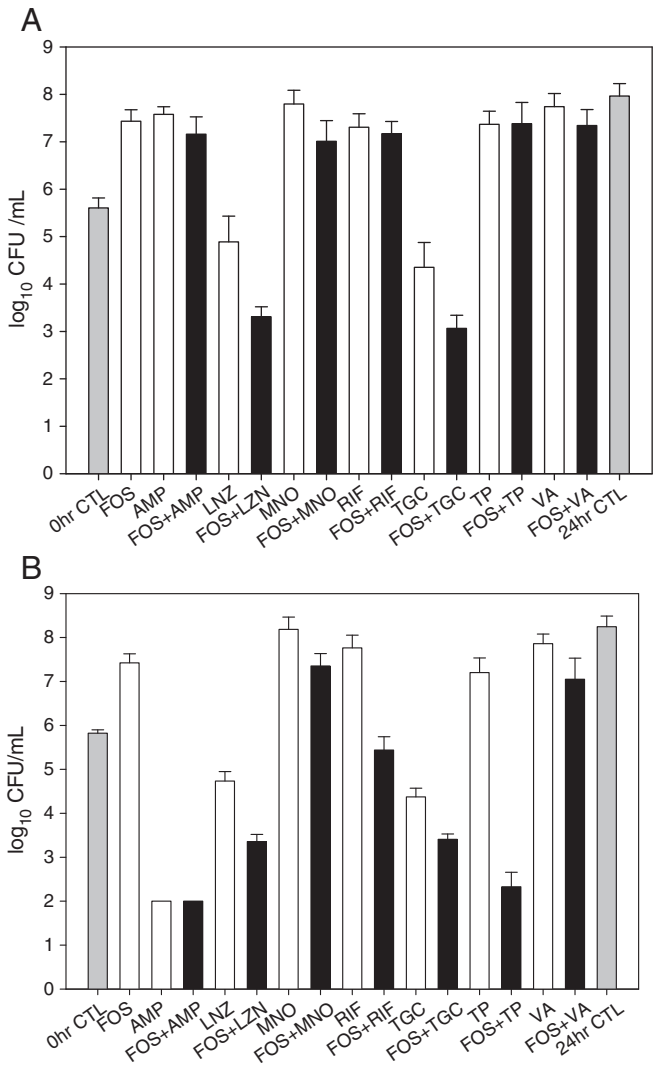

Fig. 1. Bacterial loads of VR *E. faecium* (A, 10 isolates) and VR *E. faecalis* (B, 9 isolates) with an initial inoculum of 8×10^5 CFU/mL co-cultivated with fosfomycin (FOS, 64 μg/mL), ampicillin (AMP, 8 μg/mL), linezolid (LNZ, 2 μg/mL), minocycline (MNO, 4 μg/mL), rifampicin (RIF, 1 μg/mL), tigecycline (TGC, 0.5 μg/mL), teicoplanin (TEC, 8 μg/mL), vancomycin (VA, 4 μg/mL), or fosfomycin-based combinations in the Mueller-Hinton broth at 24 h. The data are shown as the means ± SDs. The control group (CTL) was the broth containing the tested bacterium only.