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Bactericidal activity of ceftobiprole combined with different antibiotics against selected Gram-positive isolates

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ABSTRACT

This study investigated the *in vitro* susceptibility of ceftobiprole and its potential synergistic activity in combination with other antimicrobials against 46 selected Gram-positive pathogens displaying resistance or decrease susceptibility to several drugs. The gradient-cross method was used to assess synergism between ceftobiprole and daptomycin, levofloxacin, linezolid, rifampicin and piperacillin/tazobactam. Time-kill curves were performed for seven representative isolates. Ceftobiprole MICs ranged from 0.25–6 mg/L for staphylococci; 4–≥32 mg/L for *Enterococcus faecalis*, and 0.38–≥32 mg/L for *E. faecium*. Ceftobiprole *plus* daptomycin was synergistic against all isolates. Ceftobiprole *plus* linezolid was synergistic against 4 isolates belonging to different species. Ceftobiprole *plus* levofloxacin was synergistic only against enterococci. In conclusion, ceftobiprole exhibited a potent *in vitro* antibacterial activity and exhibited synergy with daptomycin against all Gram-positive isolates, despite their antibiotic resistance phenotypes. The use of ceftobiprole in combination may provide a promising alternative therapy for the treatment of resistant Gram-positive infections.

1. Introduction

Ceftobiprole is an advanced broad-spectrum cephalosporin that binds to multiple penicillin-binding proteins (PBPs), including PBP2a, and is therefore active against methicillin-resistant (MR) staphylococci (Lovering et al., 2012). Ceftobiprole medocaril, the prodrug form, has been approved in the EU and in several non-European countries for the treatment of hospital-acquired pneumonia (HAP), excluding ventilator-associated pneumonia (VAP), and for community-acquired pneumonia (CAP) caused by susceptible Gram-positive and Gram-negative pathogens, including MR *Staphylococcus aureus* (MRSA) (Syed, 2014). Ceftobiprole has demonstrated potent bactericidal activity against coagulase-negative staphylococci (CoNS), both methicillin-susceptible (MS) and MR, and against *S. aureus* (Deitchman et al., 2016).

Community-acquired pneumonia (CAP) represents one of the major cause of morbidity, mortality and healthcare costs, and one of the leading cause of hospital admission especially at the Intensive Care Unit (ICU) (Bender and Niederman, 2016). CAP may be treated with monotherapy or combination therapy, even if the potential advantage of combination therapy represents one of the controversial issues in its antibiotic therapy (Pereira et al., 2018).

Hospital-acquired pneumonia (HAP) is associated with significant mortality and has been reported to account for >25% of all infections in intensive care units (Barbier et al., 2013; Torres et al., 2009). In particular, ventilator-associated pneumonia (VAP) dramatically increase both the hospital length of stay, the cost of care and the mortality rate in patients requiring mechanical ventilation (37.7%) (Koulenti et al., 2009). Initial empirical antimicrobial therapy for HAP needs to target a broad spectrum of Gram-positive and Gram-negative pathogens. The choice of therapeutic agent should take into consideration the types and susceptibility patterns of local pathogens (including those within a particular hospital), as well as other variables such as the duration of previous hospital stay (*e.g.* for differentiation between early- and late-onset HAP), recent use of antibiotic therapy and the presence of comorbidities (Eccles et al., 2014; Torres et al., 2017). Treatment for all indications may be monotherapy, but due to their greater coverage, antibiotic combinations are frequently used as empiric therapy. In the current study, the activity and potential synergistic advantage of ceftobiprole in combination with various antibiotics was investigated using a selected group of previously characterized drug-resistant Gram-positive pathogens displaying resistance or decrease susceptibility to several drugs.

2. Materials and methods

2.1. Bacterial isolates

A total of 46 clinical- and genetically-characterized Gram-positive isolates (*S. aureus*, n = 17; CoNS spp., n = 16; *Enterococcus* spp., n = 13)

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from the Medical-Molecular Microbiology and Antibiotic-resistance Laboratory (MMARL) (Italy), were included for susceptibility and synergy testing. They displayed resistance or decrease susceptibility to several drugs: 6 methicillin-susceptible *Staphylococcus aureus* (MSSA), 5 methicillin-resistant vancomycin-susceptible *S. aureus* (MRSA/VSSA), 5 methicillin-resistant heterogeneous vancomycin intermediate *S. aureus* (MRSA/hVISA), 1 MRSA with Penicillin-Binding Protein 2a (PBP2a) mutation (Lys191Glu); 5 methicillin-susceptible *S. epidermidis*; 5 methicillin-resistant *S. epidermidis;* 5 *S. epidermidis* MDR-linezolid-resistant (LNZ-R); 6 *Enterococcus faecium,* including 3 vancomycin-resistant (VRE) and 2 β-lactamase producing (Bla^+) and 7 *E. faecalis*, including 4 VRE.

Available demographics and the molecular characterization of each isolate are given in Suppl. Table 1.

2.2. Antimicrobial susceptibility testing

MICs were determined by broth microdilution (BMD) and interpreted according to the EUCAST clinical breakpoints (http://www.eucast.org/clinical_breakpoints/) (The European Committee on Antimicrobial Susceptibility Testing, 2018). Ceftobiprole was provided by Basilea Pharmaceutica International Ltd. (Basel, Switzerland); daptomycin (Novartis, Basel, Switzerland), linezolid (Pfizer Inc., New York, NY, USA), levofloxacin, rifampicin and piperacillin/tazobactam were purchased commercially (Sigma Chemical Co., St. Louis, MO, USA).

2.3. Gradient susceptibility and synergy testing

MIC values for all antibiotics tested were determined by gradient test (GT) and were performed in duplicate according to the manufacturer's recommendations (Liofilchem®, Roseto degli Abruzzi, Italy). Synergy testing of ceftobiprole in combination with daptomycin, levofloxacin, linezolid, rifampicin and piperacillin/tazobactam was performed in duplicate using the gradient-cross or 90°-angle method. Briefly, MIC test strips were placed on Mueller–Hinton agar medium in a cross formation, with a 90° angle at the intersection between the scales at the respective minimal inhibitory concentrations (MICs) for both antimicrobial agents, as previously described (White et al., 1996). When the MIC exceeded the concentration range on one or both of the gradient strips, the strips were crossed at the highest concentration present on the respective test strip. The plates were then incubated for 18 h at 35 °C. The fractional inhibitory concentration index (FIC index) (Pillai et al., 2005) was used to interpret the gradient-cross method as follows: synergy, FIC index ≤0.5; additivity, FIC index 0.5–1; indifference, FIC index 1–4; antagonism, FIC index >4 (Fig. 1 Suppl).

2.4. Time-kill experiments

7 strains were selected for *in vitro* time-kill experiments with the same antibiotic combinations: 4 *S. aureus* isolates (MSSA, MRSA/VSSA, MRSA/hVISA and MRSA with a PBP2a mutation); 1 MDR-linezolid-resistant *S. epidermidis* isolate; 1 VRE β-lactamase-producing *E. faecium* isolate, and 1 VRE *E. faecalis* isolate.

These 7 strains primarily showed indifference to the antibiotic combinations when tested by the gradient-cross test method. The time-kill experiments were performed in duplicate in 20 mL tubes containing Cation-adjusted Mueller-Hinton broth (CA-MHB) (Difco, Detroit, MI) using a starting inoculum of 10^5–10^6 CFU/mL, with ceftobiprole (1×, 2× and 4× MIC) alone or in combination (second antibiotic at 1× MIC). All experiments were repeated at least three times, and results of a representative experiment are presented; data points are averages from duplicate CFU/ml determinations within an experiment.

Bactericidal activity was defined as a ≥3 $\log_{10}$ decrease in bacterial count at 24 h. Synergy was measured at 24 h and was defined as a ≥ 2 $\log_{10}$ decrease in CFU/mL by the combination compared with its most active constituent and a ≥ 2 $\log_{10}$ decrease in the CFU/mL below the starting inocula.

3. Results

3.1. Antimicrobial susceptibility and synergy testing

The MIC ranges and resistance rates for ceftobiprole and comparator antimicrobial agents against the 46 Gram-positive isolates are shown in Table 1. The distribution of interaction results in this diverse subset of isolates is shown in Table 2.

A total of 230 ceftobiprole combinations were performed: synergy and/or additivity were observed in 103 tests and indifference in 127 tests. Antagonism was not observed for any combinations (Table 2; Suppl. Table 2). Ceftobiprole in combination with daptomycin, linezolid and piperacillin-tazobactam represented the most active combinations

Table 1
Activity of ceftobiprole and comparator antimicrobial agents against the 46 Gram-positive isolates.

	Compound	Range (mg/L)	MIC_{50}	MIC_{90}	% (n) R
S. aureus (N = 17)	Ceftobiprole	0.25–6 (0.5–4 BMD)	0.75 (0.5 BMD)	2 (2 BMD)	5.8 (1)
	Daptomycin	0.5–3	0.75	1.5	11.7 (2)
	Levofloxacin	0.25–≥32	0.5	≥32	47 (8)
	Linezolid	1.5–8	2	3	5.8 (1)
	Rifampicin	0.004–≥32	0.012	4	17.3 (3)
	Piperacillin-tazobactam*	1–≥256	32	≥256	64.7 (11)
CoNS (N = 16)	Ceftobiprole	0.25–6 (0.06–8 BMD)	0.75 (1 BMD)	2 (2 BMD)	-
	Daptomycin	0.5–6	1.5	2	43.7 (7)
	Levofloxacin	0.19–≥32	≥32	≥32	75 (12)
	Linezolid	1–≥256	3	64	37 (6)
	Rifampicin	<0.016–≥32	0.016	≥32	31 (5)
	Piperacillin-tazobactam*	0.19–≥256	0.5	≥256	100 (16)
E. faecium (N = 6)	Ceftobiprole	4–≥32 (8–≥32 BMD)	≥32 (≥32 BMD)	≥32 (≥32 BMD)	-
	Daptomycin	3–8	3	6	-
	Levofloxacin	6–≥32	≥32	≥32	100 (6)
	Linezolid	2–≥256	3	4	20 (1)
	Rifampicin	0.016–≥32	3	≥32	-
	Piperacillin-tazobactam*	≥256	≥256	≥256	100 (6)
E. faecalis (N = 7)	Ceftobiprole	0.38–≥32 (0.5–≥32 BMD)	2 (2 BMD)	≥32 (≥32 BMD)	-
	Daptomycin	0.75–6	2	6	-
	Levofloxacin	3–≥32	≥32	≥32	86 (6)
	Linezolid	2–6	4	4	14 (1)
	Rifampicin	0.023–≥32	3	12	-
	Piperacillin-tazobactam*	4–≥256	≥256	≥256	71 (5)

* Piperacillin-tazobactam resistance was inferred from cefoxitin results for staphylococci, and from ampicillin for enterococci, as recommended by EUCAST guidelines (11).

Table 2
Interaction data obtained by gradient-cross method.

Isolate/Phenotype (N)	DAP + BPR			LEV + BPR			LNZ + BPR			RIF + BPR			TZP + BPR		
	SYN	ADD	IND	SYN	ADD	IND	SYN	ADD	IND	SYN	ADD	IND	SYN	ADD	IND
MSSA (6)	4 SYN + ADD		2	4 SYN + ADD		2	5 SYN + ADD		1	2		4	3 SYN + ADD		3
MRSA/hVISA-MDR (5)		2	3		1	4		2	3		2	3	1		4
MRSA/VSSA (5)		4	1		3	2		3	2		3	2		4	1
MRSA (mutation in PBPa) (1)			1	-		1	-		1		1	-	-		1
MSSE (5)		3	2	2 SYN + ADD		3	4 SYN + ADD		1	4 SYN + ADD		1	5		-
MRSE - MDR (6)	3		3	-		6	4 SYN + ADD		2		2	4	4		2
LNZ-R *Staphylococcus* spp. (5)	5		-	-		5	4 SYN + ADD		1	-		5	1		4
non-MDR *E. faecalis* (2)		2	-		1	1	2 SYN + ADD		-	2		-	2 SYN + ADD		-
MDR *E. faecalis* (5)	-		5		1	4	1		4		2	3	2		3
non-MDR *E. faecium* (1)	-		1	-		1	-		1	-		1	-		1
MDR *E. faecium* (5)		1	4	-		5	-		5		1	4		1	4
Total	24		22	12		34	25		21	19		27	23		23

BPR = ceftobiprole; DAP = daptomycin; LEV = levofloxacin; LNZ = linezolid; RIF = rifampicin; TZP = piperacillin/tazobactam; SYN = synergy; ADD = additivity; IND = indifference; MSSA = methicillin-susceptible *S. aureus*; MRSA = methicillin-resistant *S. aureus;* MSSE = methicillin-susceptible *S. epidermidis;* MRSE = methicillin-resistant *S. epidermidis;* MDR = multi-drug resistant.

(50–54% synergistic and additive observations). Of the synergistic results, most were observed for MSSA, but were also seen with several isolates of *S. epidermidis* and linezolid-resistant staphylococci. Among enterococci, *E. faecium* showed indifference to almost all combinations, while largely synergistic effects were seen in *E. faecalis* with ceftobiprole in combination with linezolid, rifampicin and piperacillin-tazobactam (Table 2; Suppl. Table 2).

3.2. Time-kill curve analysis

Ceftobiprole exhibited its bactericidal effect against all *Staphylococcus* spp. after 24 h, at concentrations 2× and 4× above the MIC value. The drug was active against the VRE β-lactamase-producing *E. faecium* isolate after 24 h at the 2× MIC concentration and against the VRE *E. faecalis* isolate after 8 h at 4× MIC. These data confirm the bactericidal activity of ceftobiprole against all seven selected antibiotic-resistant isolates, regardless of their species and phenotype.

In combination, ceftobiprole showed a high synergistic effect when tested in association with the anti-Gram-positive compounds in this study (Table 3).

Contrary to the observation of indifference obtained by gradient cross method, ceftobiprole plus daptomycin represented the most potent combination in the time-kill model, with synergy against all isolates at the 1× MIC of ceftobiprole (Fig. 1) and 1 $\log_{10}$ CFU/ml reduction at the 2× and 4× MICs.

The combination of ceftobiprole plus linezolid was synergistic against 4 isolates belonging to different species (MRSA/VSSA, *S. epidermidis*, *E. faecium; E. faecalis*) and showed 1 $\log_{10}$ CFU/ml reduction at the 2× and 4× MIC concentrations.

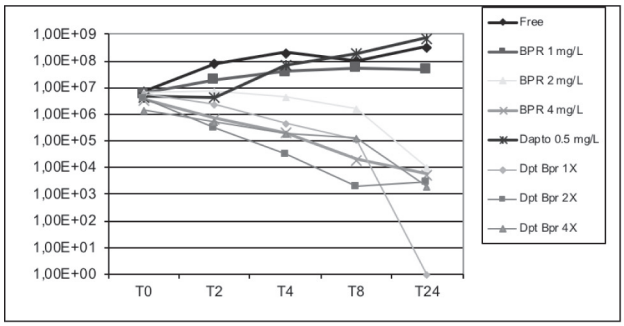
Against enterococci, the combination with levofloxacin was shown to be synergistic at the 1× ceftobiprole MIC for both *Enterococcus* species and with variable time-kill profiles at higher concentrations of ceftobiprole.

Ceftobiprole plus piperacillin-tazobactam or rifampicin produced inconsistent activities against isolates exhibiting various phenotypes: the combination with piperacillin-tazobactam was synergistic against

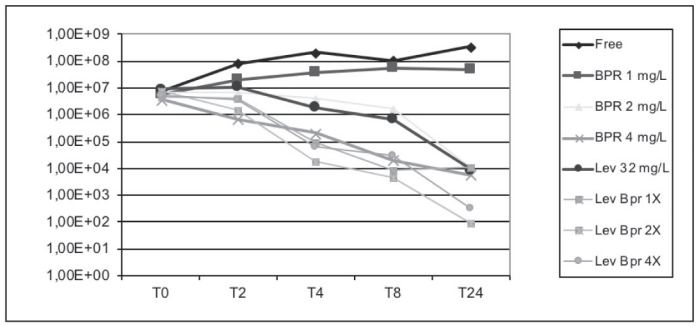
Table 3
Interaction data obtained by time-kill curve analysis.

Combination	Fold BPR MIC	MSSA	MRSA/VSSA ST8-SCCmec IV	MRSA/hVISA ST8-SCCmec IV	MRSA mut. PBP2a	*S. epidermidis* LNZ-R	*E. faecalis* VRE	*E. faecium* VRE β-lac+
		BPR 0.5 mg/L	BPR 1 mg/L	BPR 2 mg/L	BPR 4 mg/L	BPR 4 mg/L	BPR 32 mg/L	BPR 32 mg/L
		DAP 1 mg/L	DAP 0.5 mg/L	DAP 0.5 mg/L	DAP 2 mg/L	DAP 1 mg/L	DAP 1 mg/L	DAP 2 mg/L
DAP + BPR	1×	SYN	SYN	SYN	SYN	SYN	SYN	SYN
	2×	IND	IND	IND	IND	IND	SYN	IND
	4×	IND	IND	IND	SYN	IND	IND	IND
		LEV 0.5 mg/L	LEV 32 mg/L	LEV 32 mg/L	LEV 32 mg/L	LEV 32 mg/L	LEV 32 mg/L	LEV 4 mg/L
LEV + BPR	1×	IND	IND	IND	IND	IND	SYN	SYN
	2×	IND	SYN	IND	IND	IND	SYN	IND
	4×	IND	IND	IND	IND	IND	IND	SYN
		LNZ 2 mg/L	LNZ 2 mg/L	LNZ 2 mg/L	LNZ 1 mg/L	LNZ 64 mg/L	LNZ 4 mg/L	LNZ 2 mg/L
LNZ + BPR	1×	IND	SYN	IND	IND	SYN	SYN	SYN
	2×	IND	IND	IND	IND	IND	SYN	IND
	4×	IND	IND	IND	IND	IND	IND	IND
		RIF 0.12 mg/L	RIF 0.008 mg/L	RIF 0.008 mg/L	RIF 4 mg/L	RIF 32 mg/L	RIF 2 mg/L	RIF 0.016 mg/L
RIF + BPR	1×	IND	SYN	IND	IND	IND	IND	IND
	2×	IND	IND	IND	IND	IND	IND	IND
	4×	IND	IND	IND	IND	IND	IND	IND
		TZP 1 mg/L	TZP 64 mg/L	TZP 256 mg/L	TZP 256 mg/L	TZP 256 mg/L	TZP 8 mg/L	TZP 256 mg/L
TZP + BPR	1×	IND	IND	SYN	IND	SYN	IND	IND
	2×	IND	IND	IND	IND	IND	IND	IND
	4×	IND	IND	IND	IND	IND	IND	IND

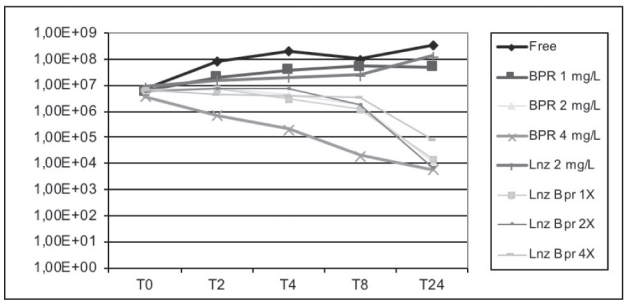
SYN = synergy; IND = indifference; BPR = ceftobiprole; DAP = daptomycin; LEV = levofloxacin; LNZ = linezolid; RIF = rifampicin; TZP = piperacillin/tazobactam.
Synergy was defined as a ≥ 2 log10 CFU/mL decrease in viable counts with the combination as compared with its most active single agent after 24 h of incubation, and the number of surviving organisms in the presence of the combination was ≥2 log 10 CFU/mL below the starting inoculum.
Combination drugs (DAP-LEV-LNZ-RIF-TZP) tested at 1× MIC.



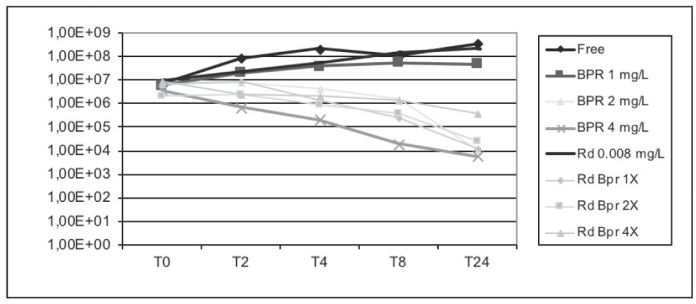
MIC, minimum inhibitory concentration; BPR, ceftobiprole (1× = MIC 1 mg/L); DPT, daptomycin (1× = MIC 0.5 mg/L).



MIC, minimum inhibitory concentration; BPR, ceftobiprole (1× = MIC 1 mg/L); Lev, levofloxacin (1× = MIC 32 mg/L).



MIC, minimum inhibitory concentration; BPR, ceftobiprole (1× = MIC 1 mg/L); Lnz, linezolid (1× = MIC 2 mg/L).



MIC, minimum inhibitory concentration; BPR, ceftobiprole (1× = MIC 1 mg/L); Rd, rifampicin (1× = MIC 0.008 mg/L).

Fig. 1. Examples of time-kill curves for the MRSA/VSSA ST8-SCC*mec* IV isolate.

S. epidermidis and, notably, against the MRSA/hVISA isolate; combination with rifampicin produced effects only against the MRSA/VSSA isolate, with a 3 $\log_{10}$ CFU/mL reduction.

4. Discussion

The current investigation examined the *in vitro* antibacterial activity, in terms of inhibition and killing, of ceftobiprole alone and in combination with different anti-Gram-positive drugs against a challenging group of staphylococci and enterococci.

Ceftobiprole exhibited good *in vitro* antibacterial activity against all Gram-positive isolates tested.

The gradient-cross test has proved to be a rapid and easy-to perform method, to evaluate the combination of ceftobiprole with different antimicrobial agents; even if it is well known that time-kill curve analysis remains the gold standard, it should be suggested as a preliminary test for the evaluation of the synergistic combinations in the hospital setting.

The gradient-cross test combination data obtained in the present study suggest that synergism with ceftobiprole was common against MSSA; however, no synergy (additivity at best) was observed against MRSA. It is likely that in these assays, synergistic effects were masked by the high activity of ceftobiprole alone against these strains, a conclusion that is supported by the time-kill results. For *S. epidermidis*, synergy occurred for both MSSE and MRSE but was more common for the MSSE strains tested. It is noteworthy, that although only 5 linezolid-resistant staphylococci were tested, synergy or additivity for ceftobiprole combinations with daptomycin was observed in 2 and 3 isolates, respectively. Even with the less sensitive test, synergy was detected among these isolates, suggesting that a greater synergistic magnitude through time-kill experiments may be detected.

The *in vitro* time evaluation of the antibiotic effect with different starting concentrations, performed by time-kill curve experiments, appears to be more sensitive with respect to gradient-cross method, being able to detect the synergistic effect among the antibiotics studied. The more classical time-kill experiments were carried out using isolates for which only indifference was shown by the gradient-cross method. These isolates were selected to understand if indifferent results could be improved *via* traditional time-kill experiments. Indeed, while individually daptomycin at the MIC was not effective and ceftobiprole at 1× MIC concentration failed to attain a 3-$\log_{10}$ killing effect, combinations of the two did show bactericidal activity (*i.e.*, ≥3-$\log_{10}$ kill) and these effects were classified as synergistic (*i.e.*, ≥ 2-$\log_{10}$ kill beyond that achieved by ceftobiprole). In most cases, the combination attained at least a 3-$\log_{10}$ killing effect.

The data from the present study are consistent with the synergistic antimicrobial killing activity observed with daptomycin *plus* β-lactam antibiotics against MRSA (Chambers et al., 2016; Oltolini et al., 2016). In a separate study that also examined the *in vitro* activity of ceftobiprole *plus* daptomycin, the combination was synergistic against MRSA strains of differing vancomycin and daptomycin susceptibilities. When sub inhibitory concentrations of ceftobiprole were added, the MICs of daptomycin and vancomycin were consistently reduced across multiple strains tested. In this study, growth reduction was seen also with the combination of daptomycin *plus* ceftobiprole in standard time-kill curve studies for all strains tested (Barber et al., 2014). Furthermore, ceftobiprole has demonstrated promising activity both alone and in combination against biofilms of MS- and MR-staphylococci at clinically-relevant concentrations (Abbanat et al., 2014), which is important as many infections *in situ* are more biofilm-like in nature.

The gradient-cross test for enterococci showed synergistic and additivity effect in 18/65 (27.6%) combinations and indifferent effect in all others. Other studies have also shown that ceftobiprole and ampicillin both caused an increase in the activity of daptomycin against daptomycin-susceptible and daptomycin-resistant VRE and in time-kill studies, daptomycin plus either ceftobiprole or ampicillin was synergistic against 4/6 of the VRE strains tested (Werth et al., 2014). Similarly, ceftobiprole alone exhibited good *in vitro* activity against *E. faecalis*, including β-lactamase producing (Bla^+) and VRE strains, and exhibited synergism with aminoglycosides against selected isolates (Arias et al., 2007).

5. Conclusions

The synergistic potential of ceftobiprole in combination with other antibiotics merits further studies. Specifically, additional time-kill studies to more fully understand the potential of supra- and sub-MIC concentrations of ceftobiprole against isolates with known mechanisms of resistance are warranted. Findings from such studies may help guide the therapeutic use of ceftobiprole in drug combination therapy especially where resistant pathogens are suspected or confirmed.

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Declarations of interests

None.
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Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2018.07.015.

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