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In Vitro Synergistic Activity of Fosfomycin–Colistin Combination Against Carbapenem–Resistant *Pseudomonas Aeruginosa* from Ventilator–Associated Pneumonia Patients

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Abstract:

Objective: Infections caused by carbapenem–resistant *Pseudomonas aeruginosa* (CRPA) are a global health problem due to its multidrug resistance, often leading to treatment failure. This study aimed to assess the synergistic activity of fosfomycin plus colistin combination against clinical CRPA isolates from ventilator–associated pneumonia (VAP) patients.

Material and Methods: This cross–sectional study retrospectively collected clinical data on 40 VAP patients with CRPA infections in 2023. CRPA clinical isolates were obtained between 2022 and 2023 from the sputum of VAP patients as part of the carbapenem–resistant isolate collection. The susceptibility to carbapenems, fosfomycin, and colistin was evaluated on CRPA isolates using the broth microdilution method. The synergistic activity of fosfomycin and colistin combination against CRPA isolates was assessed using the checkerboard assay. The time–kill study was then conducted on CRPA isolates that exhibited treatment synergism with the combination.

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Results: The most common comorbidities were chronic pulmonary disease and diabetes mellitus, which were found in 73% and 70% of VAP patients with CRPA infection. Previous carbapenem exposure was observed in 93% of the patients. Since VAP diagnosis, these patients were on a ventilator for a median of 13 days with an interquartile range of 10–22 days. Approximately 92% and 95% of the CRPA isolates exhibited high and low carbapenem and colistin resistance, respectively. In contrast, only 3 (7%) isolates were extremely resistant to fosfomycin, classifying as the non–wild type (bacterial isolates with reduced susceptibility or resistance). Synergism between fosfomycin and colistin was only observed in 2 (5%) isolates. Time–kill kinetics of these 2 isolates revealed a ≥2–log reduction in 1⁄4 minimum inhibitory concentration of fosfomycin and colistin.

Conclusion: *In vitro* findings indicate that the synergistic activity of fosfomycin combined with colistin against CRPA isolates was rare. While this combination showed potential activity in these few isolates, further studies are needed to determine its clinical relevance and effectiveness in treating severe CRPA infections.

Keywords: colistin, carbapenem–resistant *Pseudomonas aeruginosa*, fosfomycin, synergistic activity, ventilator–associated pneumonia

Introduction

Pseudomonas aeruginosa is an opportunistic Gram–negative pathogen that commonly causes healthcare–associated infections, including respiratory tract infections, urinary tract infections (UTIs), bloodstream infections (BSIs), and surgical site infections[1,2]. *P. aeruginosa* is a leading cause of ventilator–associated pneumonia (VAP) in the intensive care unit (ICU) with a 30–60% mortality rate[3]. Moreover, *P. aeruginosa* has become a public health concern because of its increasing resistance to many antimicrobial classes, and multidrug–resistant (MDR) strains have been identified. In 2019, the Centers for Disease Control and Prevention listed MDR *P. aeruginosa* (MDR–PA) as a serious threat, with 32,600 estimated cases in hospitalized patients 2,700 estimated deaths, and an estimated US$767 million attributable healthcare costs in 2017[2]. Carbapenems (imipenem, meropenem, and doripenem) have been used as the last line of treatment for MDR–PA infections in the previous 2 decades[4], and carbapenem resistance has been detected in this pathogen. Some isolates of carbapenem–resistant *P. aeruginosa* (CRPA) carry carbapenemase genes on mobile genetic elements, raising concerns regarding the rapid spread of resistance to other *P. aeruginosa* isolates or other bacteria[2]. Besides carbapenemase enzyme production to inactivate carbapenems, carbapenem resistance is facilitated by efflux pump overexpression, outer membrane porin loss, AmpC β–lactamase production, or a combination of these mechanisms[5].

Fosfomycin, a phosphoenolpyruvate analog, was discovered in 1969 and is frequently used to treat uncomplicated UTIs, especially acute cystitis[6]. It also has roles in respiratory infections in cystic fibrosis, osteomyelitis, and bacterial meningitis[6]. Additionally, it is used as an alternative for complicated UTIs, prostatitis, and MDR infections like extended–spectrum β–lactamase–producing *Escherichia coli (E. coli)*, carbapenem–resistant Enterobacterales (CRE), and vancomycin–resistant *Enterococcus* spp. (VRE)[6]. Fosfomycin interrupts the first cytoplasmic bacterial cell wall synthesis step by binding to UDP–N–acetylglucosamine enolpyruvyltransferase, inhibiting peptidoglycan production[7]. In hospital settings, fosfomycin is most effective in young women

with uncomplicated UTIs and patients with MDR infections, while it also has the potential to treat immunocompromised patients. However, it is less effective for severe systemic infections due to poor tissue penetration, resulting in inappropriate monotherapy for pyelonephritis and BSIs caused by *P. aeruginosa* or *Acinetobacter baumannii*[6]. Fosfomycin also has several limitations, particularly in patients with severe comorbidities and in cases of prolonged use[6]. Pathogens can rapidly develop resistance to fosfomycin with prolonged or repeated use, limiting its long-term effectiveness[6]. Fosfomycin resistance in Gram-negative and Gram-positive pathogens, especially MDR-PA, CRE, and VRE, is becoming more prevalent, especially in regions with high rates of infection caused by MDR pathogens. Resistance varies by region, with some areas showing low resistance and others reporting significant increases[8,9]. Mechanisms of fosfomycin resistance mainly include mutations in transport or uptake proteins, modification of the fosfomycin target MurA, and fosfomycin modification[8,9]. In addition, oral fosfomycin has variable bioavailability, and its intravenous (IV) formulation is not widely available in many regions[6]. Another challenge of fosfomycin use is its reduced effectiveness in specific circumstances, such as in the presence of high bacterial load or infections caused by strains with reduced susceptibility to fosfomycin (non-wild-type strains)[6]. According to those challenges mentioned above, most studies suggest that the combination of fosfomycin with other antimicrobial agents can be effective against many pathogens, especially MDR-PA[6,10,11].

Colistin (also known as polymyxin E), a polycationic peptide antimicrobial, was discovered in 1949 and is considered the last-line treatment for Gram-negative MDR pathogens, particularly carbapenem-resistant strains[12]. Colistin binds to the phosphate groups (anionic molecules) of lipid A, a crucial lipopolysaccharide (LPS) structure, by displacing divalent calcium and magnesium cations from the outer cell membrane[12,13]. Lipid A is a crucial LPS that plays an important role in bacterial permeability. Thus, this electrostatic interaction results in changes in permeability, and the cell contents consequently leak. Colistin resistance and hetero-resistance in *P. aeruginosa* and other Gram-negative bacteria, such as *E. coli*, have been observed in several regions, raising concern and complicating treatment strategies[14-17]. A systematic review by Narimisa et al. (2024) revealed that colistin resistance in *P. aeruginosa* increased from 2% to 5% from 2006 to 2023[17]. Various resistance mechanisms, including chromosomal mutations and plasmid-mediated *mcr* gene acquisition, exacerbate the threat of colistin resistance in these pathogens[18,19]. Lin et al. (2019) reported that resistance and heteroresistance to colistin in *P. aeruginosa* are primarily associated with alterations in the *pmrA* and *pmrB* genes encoding a regulatory system[18]. In the clinical setting, the colistin resistance rate increased from 1% to 7% in cystic fibrosis patients[17]. Colistin resistance is also associated with treatment failures and increased mortality, particularly in infections caused by *A. baumannii* and other Gram-negative bacteria[20]. Moreover, inadequate colistin plasma levels contribute to poor patient outcomes[21]. Colistin resistance, particularly in infections caused by MDR pathogens, highlights the need for alternative therapies. As colistin is a last-resort treatment for pathogens like *P. aeruginosa* and *A. baumannii*, its growing resistance demands new strategies. Combination therapies, such as fosfomycin and colistin, offer promise by enhancing colistin's efficacy through synergistic action and targeting different bacterial pathways. However, although fosfomycin is not routinely recommended for *Pseudomonas* spp. Infections, according to the Clinical Laboratory Standard Institute (CLSI) guidelines, and the synergistic effects of its combination with colistin have been previously reported. This combination has shown enhanced bacterial killing and reduced resistance development in CRPA isolates, as supported by previous

studies[22,23]. Some *in vitro* studies reported synergy in 13% – 21% of isolates, while clinical studies suggest improved microbiological response rates without significant mortality benefits[22,23]. This approach not only facilitates overcoming resistance but also reduces the risk of further resistance development, ultimately improving patient outcomes in infections caused by highly resistant pathogens, especially CRPA clinical isolates.

In the present study, we hypothesized that antimicrobial resistance (AMR) in patients with VAP may lead to some clinical characteristics such as severe infection, treatment difficulty, and high complication and mortality rates. Furthermore, due to the inconsistent prior findings, rapidly developing resistance mechanisms, and the need for effective alternative treatments for CRPA infection, exploring the potential efficacy of the fosfomycin–colistin combination against CRPA clinical isolates remains necessary. Hence, we aimed to assess the synergistic activity of fosfomycin in combination with colistin against clinical CRPA isolates from VAP patients admitted at Songklanagarind Hospital, Thailand.

Material and Methods

Clinical data and bacterial isolates

The study was conducted in accordance with the Declaration of Helsinki and approved by the Human Research Ethics Committee (HREC) of Prince of Songkla University (protocol code: 64–284–14–1). The study was determined to be exempt from full ethical review based on the criteria outlined by the HREC. Our cross–sectional study retrospectively collected clinical data on 40 VAP patients with CRPA infections, and 40 non–duplicated CRPA isolates were provided by the Clinical Microbiology Laboratory of Songklanagarind Hospital, Songkhla, Thailand, in 2023. All 40 CRPA isolates were collected from the sputum of VAP patients between July 1, 2022, and July 31, 2023, as part of the sampling method for carbapenem–resistant isolates. VAP is a nosocomial pneumonia that occurs ≥48 hours after endotracheal intubation and mechanical ventilation and its diagnosis is based on clinical signs (fever, leukocytosis, purulent secretions, lung infiltrates) and microbiological confirmation via endotracheal aspirate and bronchoalveolar lavage cultures ($\geq 10^4$–10^5 CFU/mL). Regarding the details of bacterial isolation, sputum specimens obtained from VAP patients were streaked onto selective media for bacterial isolation. The isolates were then identified through biochemical testing and further confirmed using a Matrix–Assisted Laser Desorption/Ionization Time–of–Flight Mass Spectrometer[24]. Carbapenem resistance in the isolates was initially investigated using a disk diffusion method, according to the CLSI guidelines (33rd edition, 2023)[25]. The isolates resistant to at least one carbapenem antibiotic (imipenem, meropenem, and/or doripenem) were classified as carbapenem–resistant. These 40 isolates were obtained from 25 (63%) male patients and 15 (37%) female patients, aged 25 to 97 years, all showing signs of pneumonia.

Broth microdilution assay

The minimum inhibitory concentrations (MICs) of meropenem, imipenem, doripenem, fosfomycin, and colistin were determined using the broth microdilution method. CRPA clinical isolates were cultured at 37 °C and 150 rpm for 4 to 6 hours to prepare the log phase of bacterial growth. The concentration of the bacteria was adjusted to 0.5 McFarland standard (approximately 1×10^8 CFU/mL) and continually diluted 1:100 (approximately 1×10^6 CFU/mL). Meanwhile, a two–fold serial dilution of each antibiotic was prepared in 96–well microtiter plates at a final volume of 100 µL. Afterward, 100 µL CRPA solution was added to the wells, and the plates were incubated at 37 °C for 16–20 hours. The MICs were assessed using the resazurin test, and the susceptible, intermediately resistant,

and resistant results of all the tested antimicrobial agents, except fosfomycin, were interpreted according to the CLSI guidelines (33rd edition, 2023)[25]. The MIC breakpoints for meropenem, imipenem, and doripenem were defined as ≤2 µg/mL (susceptible), 4 µg/mL (intermediate), and ≥8 µg/mL (resistant). For colistin, the MIC breakpoints were ≤4 µg/mL (intermediate–resistant) and ≥8 µg/mL (resistant). Fosfomycin susceptibility was interpreted according to the European Committee on Antimicrobial Susceptibility Testing guideline (Version 9.0, 2019)[26]. Fosfomycin MIC values of ≤256 µg/mL were interpreted as wild–type isolates, while values >256 µg/mL were considered non–wild–type isolates. The wild–type phenomenon for fosfomycin is defined as susceptible bacterial isolates with no acquired resistance mechanisms. In contrast, the non–wild–type is defined as bacterial isolates with reduced susceptibility or resistance. The *P. aeruginosa* ATCC27853 was used for quality control and the experiments were duplicated.

Checkerboard assay

The synergistic activity of fosfomycin and colistin was investigated using a checkerboard assay. The log phase of the bacterial solution was prepared as previously described. Meanwhile, two–fold serial dilutions of fosfomycin and colistin were prepared and mixed in the 96–well microtiter plates at a final volume of 100 µL. Afterward, 100 µL CRPA solution was added to the wells, and the plates were incubated at 37 °C for 16–20 hours. Bacterial inhibition was observed using the resazurin test, and the synergistic activities were assessed by calculating a fractional inhibitory concentration index (FICI), as previously described[27]. The experiment was conducted in 3 replicates:

The FICI for each combination was interpreted as follows: FICI ≤0.5, synergism; 0.5 <FICI <1, additive; 1 ≤FICI <4, indifference; FICI ≥4, antagonism. The experiment was duplicated.

Time–kill assay

The reduction or growth of a bacterial population after exposure to the antimicrobial at different time intervals was measured using the time–kill assay. Here, the time–kill kinetics of representative CRPA clinical isolates exhibiting the synergistic, indifferent, and antagonistic effects of the fosfomycin–colistin combination were studied in order to assess bacteriostatic (inhibition of bacterial growth without killing), bactericidal (killing bacteria), and synergistic (enhanced effect when 2 or more antimicrobials work together) activities. The log phase of the bacterial solution was prepared as previously described. The ranges of antibiotic concentrations individually and in combination were designed and prepared using cation–adjusted Muller–Hinton broth in culture tubes at a final volume of 10 mL. Afterward, 10 mL of representative CRPA solution was added to the tubes, which were then incubated at 37 °C for 0–24 hours. An untreated tube was used as a growth control. Ten microliters were pipetted from each tube at zero, 2, 4, 8, 12, and 24 hours of incubation and dropped onto Tryptic Soy Agar plates. The plates were incubated at 37 °C for 16–20 hours and the number of colonies was counted to calculate the CFU/mL. A ≥2–log reduction in CFU/mL compared with the most active single treatment was considered as synergism, whereas a ≥3–log reduction in CFU/mL compared with the initial colony count at time zero (0 hour) was considered as bactericidal[22,28]. The experiment was conducted in 3 replicates.

Results

Clinical characteristics of patients

Clinical characteristics of VAP patients with CRPA infection are demonstrated in Table 1. Forty CRPA clinical isolates were collected from 40 patients (one isolate per patient). The comorbidities in these patients were chronic pulmonary disease (73%), diabetes mellitus (70%), hypertension (48%), cardiovascular disease (40%), chronic kidney disease (38%), hematologic malignancy (33%), cerebrovascular disease (25%), immunocompromised (20%), and/or solid organ malignancy (18%). A Charlson comorbidity index (CCI) score >3 was recorded in 35 (87%) patients, with a median CCI score of 7 (interquartile range [IQR]: 5-9). Notably, 93%, 80%, 75%, 63%, and 43% of the patients had been previously treated with carbapenems, β-lactam/β-lactamase inhibitor, cephalosporins, fluoroquinolones, and aminoglycosides, respectively. Most patients (73%) were initially admitted to the ICU. In addition, 95% and 78% of patients underwent urinary catheterization and intravascular devices, respectively. The median time from mechanical ventilation to VAP was 6 days, with an IQR of 4 to 9 days. The median length of hospital stay after the end of VAP treatment was 32 days (IQR: 19-47 days), while the median number of ventilator days since the diagnosis of VAP was 13 days (IQR: 10-22 days). For the severity, a sequential organ failure assessment (SOFA) score of ≥2 was found in 36 (90%) patients, with a median SOFA score of 6 (IQR: 3-9), while septic shock was observed in 16 (40%) patients. Treatment failure was reported in 21 (52%) patients. In-hospital mortality was observed in 29 patients (73%).

Antimicrobial susceptibility profiles

The MICs of meropenem, imipenem, doripenem, fosfomycin, and colistin were evaluated against the CRPA clinical isolates using the broth microdilution method. The results showed that 98%, 95%, and 93% of the isolates were resistant to meropenem, imipenem, and doripenem, respectively, and all isolates were confirmed to be CRPA (Table 2 and Table 3). High MIC values of meropenem (MIC_{50} and MIC_{90} of 512 and >512 μg/mL, respectively), imipenem (MIC_{50} and MIC_{90} of 128 and 256 μg/mL, respectively), and doripenem (MIC_{50} and MIC_{90} of 256 and 512 μg/mL, respectively) were observed in most isolates. In addition, 93% of the isolates were resistant to colistin with low MIC values. Regarding fosfomycin, 37 (93%) isolates were considered as wild-type isolates with the MICs ranging between 32 and 256 μg/mL. The other 3 (7%) isolates, CRPA6, CRPA8, and CRPA23, were classified as non-wild-type isolates that are highly resistant to fosfomycin, with the MICs ranging between 8192 and 16,384 μg/mL.

Synergistic activity

The synergistic activity of fosfomycin and colistin was investigated against CRPA clinical isolates. An additive effect (a combined effect equals the sum of individual effects) of this combination was observed in almost all isolates (n=37, 93%), as shown in Table 4. Notably, a synergistic effect was observed in 2 isolates (CRPA9 and CRPA31), whereas an indifferent effect was observed in only one isolate (CRPA27). No antagonistic effects were observed in the isolates studied.

Time-kill kinetics

Time-kill experiments were performed to confirm the synergistic effects of fosfomycin and colistin on CRPA9 and CRPA31 isolates (Figure 1). At 12 hours, colistin at 1/4 MIC combined with fosfomycin at 1/4 MIC demonstrated synergistic effects on the CRPA9 isolate, with a 2-log decrease in CFU/mL (99% reduction) compared with the most active agent (colistin alone at 1/4 MIC). In addition, fosfomycin treatment (1/4 MIC) in combination with colistin

(1/4 MIC) for 24 h showed bactericidal effects, with a 3-log decrease in CFU/mL (99.9% reduction) compared with the most active agent. For the CRPA31 isolate, synergistic effects were observed when treated with a combination of colistin (1/4 MIC) and fosfomycin (1/4 MIC) within 4 hours. Notably, bactericidal effects were observed with a bacterial reduction of >4 log CFU/mL (>99.99% reduction) within 8 hours for the CRPA31 isolate. However, no bacterial reduction was observed upon treatment with either fosfomycin or colistin alone in the CRPA9 and CRPA31 isolates.

Table 1 Clinical characteristics of patients with carbapenem-resistant *P. aeruginosa*-induced ventilator-associated pneumonia

Parameter	Patients with VAP due to CRPA infection (N=40)
Demographics	
Median [IQR] patient age (years)	50 [42-79]
Male	25 (63%)
Female	15 (37%)
Comorbidities	
Immunocompromised	8 (20%)
Diabetes mellitus	28 (70%)
Hypertension	19 (48%)
Chronic kidney disease (s)	15 (38%)
Cardiovascular disease (s)	16 (40%)
Cerebrovascular disease (s)	10 (25%)
Chronic pulmonary disease (s)	29 (73%)
Solid organ malignancy	7 (18%)
Hematologic malignancy	13 (33%)
Median [IQR] CCI score	7 [5-9]
Previous antibiotic exposure within 3 months	
Carbapenem (s)	37 (93%)
β-lactam/β-lactamase inhibitor	32 (80%)
Cephalosporin (s)	30 (75%)
Fluoroquinolone (s)	25 (63%)
Aminoglycoside (s)	17 (43%)
Clinical characteristics	
Initial ICU admission	29 (73%)
Median [IQR] APACHE II score	17 [13-21]
Invasive medical devices	
Intra-vascular	31 (78%)
Urinary catheterization	38 (95%)
Median [IQR] of time from MV to VAP (days)	6 [4-9]
Antibiotic treatments	
Monotherapy	
Cefoperazone/Sulbactam	1 (3%)
Ceftazidime	2 (5%)
Ciprofloxacin	2 (5%)
Colistin	19 (48%)
Meropenem	1 (3%)
Piperacillin/Tazobactam	3 (8%)

Table 1 (continued)

Parameter	Patients with VAP due to CRPA infection (N=40)
Combination therapy	
Cefoperazone/Sulbactam+Ciprofloxacin	2 (5%)
Colistin+Piperacillin/Tazobactam	1 (3%)
Colistin+Ceftazidime	1 (3%)
Colistin+Cefoperazone/Sulbactam	1 (3%)
Colistin+Meropenem	3 (8%)
Colistin+Ciprofloxacin	2 (5%)
Colistin+Fosfomycin	2 (5%)
Severity	
Median [IQR] SOFA score	6 [3-9]
Septic shock	16 (40%)
Treatment failure	21 (52%)
Mortality	
14 days	21 (53%)
30 days	26 (65%)
In-hospital	29 (73%)
Median [IQR] length of hospital stay after the end of VAP treatment (days)	32 [19-47]
Median [IQR] number of ventilator days since diagnosis of VAP (days)	13 [10-22]
Median [IQR] hospital cost (baht)	245,004 [129,235-266,991]

VAP=ventilator-associated pneumonia, MV=mechanical ventilator, ICU=intensive care unit, CCI=Charlson comorbidity index, SOFA=sequential organ failure assessment, APACHE II=acute physiology and chronic health evaluation, IQR=interquartile range

Table 2 Antimicrobial susceptibility profiles of clinical carbapenem-resistant *P. aeruginosa* isolates

Isolate code	Minimum inhibitory concentration (μg/mL)				
	Meropenem[a]	Imipenem[a]	Doripenem[a]	Fosfomycin[b]	Colistin[c]
CRPA1	512	128	256	64	4
CRPA2	128	64	128	64	4
CRPA3	512	128	256	32	4
CRPA4	512	>512	256	64	4
CRPA5	512	128	256	64	4
CRPA6	256	8	128	16,384	4
CRPA7	512	128	256	64	4
CRPA8	>512	>512	512	8,192	4
CRPA9	>512	128	256	64	8
CRPA10	512	128	256	64	4
CRPA11	512	128	256	32	4
CRPA12	512	128	256	64	4
CRPA13	512	64	256	64	4
CRPA14	>512	128	512	32	4

Table 2 (continued)

Isolate code	Minimum inhibitory concentration (μg/mL)				
	Meropenem[a]	Imipenem[a]	Doripenem[a]	Fosfomycin[b]	Colistin[c]
CRPA15	>512	256	256	64	4
CRPA16	512	64	256	64	4
CRPA17	>512	256	512	32	4
CRPA18	512	64	256	64	4
CRPA19	32	32	16	64	4
CRPA20	8	1	4	128	4
CRPA21	64	16	16	64	4
CRPA22	512	64	256	128	4
CRPA23	128	32	64	16,384	4
CRPA24	512	64	256	64	4
CRPA25	>512	256	512	32	4
CRPA26	512	64	256	64	4
CRPA27	>512	128	512	32	2
CRPA28	8	4	2	64	4
CRPA29	256	128	256	32	4
CRPA30	256	128	128	64	4
CRPA31	256	256	128	64	4
CRPA32	256	256	256	128	4
CRPA33	4	8	1	256	4
CRPA34	256	128	256	64	4
CRPA35	128	64	64	64	4
CRPA36	512	256	256	128	2
CRPA37	256	256	256	128	2
CRPA38	256	128	256	64	4
CRPA39	256	256	256	256	4
CRPA40	256	256	256	128	4

[a]MIC values of ≤2 μg/mL, 4 μg/mL, and ≥8 μg/mL were classified as susceptible, intermediate, and resistant, respectively. [b]MIC values of ≤256 μg/mL and >256 μg/mL were classified as wild-type and non-wild-type isolates, respectively. [c]MIC values of ≤4 μg/mL and ≥8 μg/mL were classified as intermediate and resistant, respectively.

Table 3 Overview of antimicrobial susceptibility for clinical carbapenem-resistant *P. aeruginosa* isolates

Antibiotic	Number of the isolates (percentage)				
	Susceptible	Intermediate	Resistant	Wild-type isolates	Non-while-type isolates
Imipenem	0 (0%)	1 (3%)	39 (98%)	ND	ND
Meropenem	1 (3%)	1 (3%)	38 (95%)	ND	ND
Doripenem	2 (5%)	1 (3%)	37 (93%)	ND	ND
Fosfomycin	ND	ND	ND	37 (93%)	3 (8%)
Colistin	ND	39 (98%)	1 (3%)	ND	ND

ND=not determined

Table 4 Synergistic activity of fosfomycin and colistin combination against clinical carbapenem-resistant *P. aeruginosa* isolates

Isolate code	MIC of FOS (μg/mL)			MIC of COL (μg/mL)			FICI	Interpretation[a]
	Alone	Combination	FIC_{FOS}	Alone	Combination	FIC_{COL}		
CRPA1	64	16	0.25	4	2	0.5	0.75	Add
CRPA2	64	16	0.25	4	2	0.5	0.75	Add
CRPA3	32	8	0.25	4	2	0.5	0.75	Add
CRPA4	64	16	0.25	4	2	0.5	0.75	Add
CRPA5	64	16	0.25	4	2	0.5	0.75	Add
CRPA6	16,384	4,096	0.25	4	2	0.5	0.75	Add
CRPA7	64	16	0.25	4	2	0.5	0.75	Add
CRPA8	8,192	2,048	0.25	4	2	0.5	0.75	Add
CRPA9	64	16	0.25	8	2	0.25	0.5	Syn
CRPA10	64	16	0.25	4	2	0.5	0.75	Add
CRPA11	32	8	0.25	4	2	0.5	0.75	Add
CRPA12	64	16	0.25	4	2	0.5	0.75	Add
CRPA13	64	16	0.25	4	2	0.5	0.75	Add
CRPA14	32	8	0.25	4	2	0.5	0.75	Add
CRPA15	64	16	0.25	4	2	0.5	0.75	Add
CRPA16	64	16	0.25	4	2	0.5	0.75	Add
CRPA17	32	8	0.25	4	2	0.5	0.75	Add
CRPA18	64	16	0.25	4	2	0.5	0.75	Add
CRPA19	64	16	0.25	4	2	0.5	0.75	Add
CRPA20	128	32	0.25	4	2	0.5	0.75	Add
CRPA21	64	16	0.25	4	2	0.5	0.75	Add
CRPA22	128	32	0.25	4	2	0.5	0.75	Add
CRPA23	16,384	4,096	0.25	4	2	0.5	0.75	Add
CRPA24	64	16	0.25	4	2	0.5	0.75	Add
CRPA25	32	8	0.25	4	2	0.5	0.75	Add
CRPA26	64	16	0.25	4	2	0.5	0.75	Add
CRPA27	32	8	0.25	2	4	2	2.25	Ind
CRPA28	64	16	0.25	4	2	0.5	0.75	Add
CRPA29	32	8	0.25	4	2	0.5	0.75	Add
CRPA30	64	16	0.25	4	2	0.5	0.75	Add
CRPA31	64	16	0.25	4	1	0.25	0.5	Syn
CRPA32	128	32	0.25	4	2	0.5	0.75	Add
CRPA33	256	64	0.25	4	2	0.5	0.75	Add
CRPA34	64	16	0.25	4	2	0.5	0.75	Add
CRPA35	64	16	0.25	4	2	0.5	0.75	Add
CRPA36	128	32	0.25	2	1	0.5	0.75	Add
CRPA37	128	32	0.25	2	1	0.5	0.75	Add
CRPA38	64	16	0.25	4	2	0.5	0.75	Add
CRPA39	256	64	0.25	4	2	0.5	0.75	Add
CRPA40	128	32	0.25	4	2	0.5	0.75	Add

MIC=minimum inhibitory concentration, FOS=fosfomycin, COL=colistin, FIC=fractional inhibitory concentration, FICI=fractional inhibitory concentration index, Syn=synergism, Add=additivity, Ind=indifference, Ant=antagonism, [a]FICI ≤0.5, 0.5 <FICI <1, 1 ≤FICI <4, and FICI ≥4 were classified as synergism, additive, indifference, and antagonism, respectively.

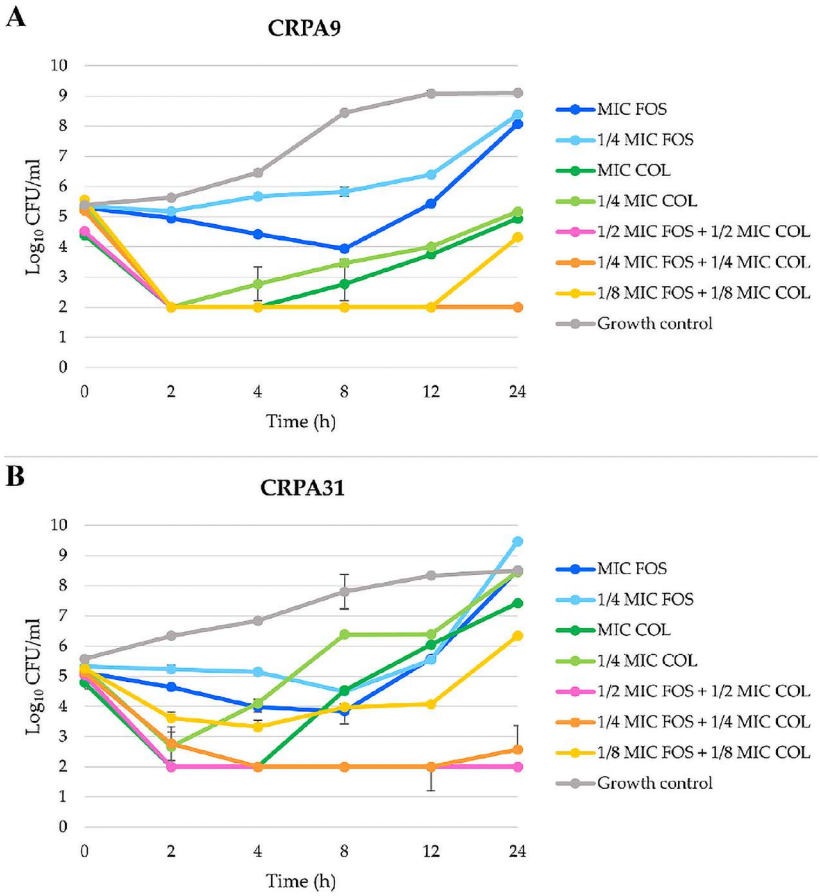

Figure 1 Time-kill kinetics of fosfomycin and colistin individually and in combination against the CRPA9 and CRPA31 isolates

Discussion

AMR reports in Thailand exhibited that the resistance rates to imipenem and meropenem in *P. aeruginosa* have been slowly increasing since 2000[29]. This trend underscores the growing challenge of AMR and highlights the need for a deeper understanding of patients with CRPA-induced VAP. Consequently, this present study investigates the characteristics of these patients and alternative treatment options.

Most patients with CRPA-induced VAP exhibited medical comorbidities, particularly chronic pulmonary disease and diabetes mellitus, were exposed to carbapenems before the CRPA infection. Prior exposure to carbapenems might be considered a risk factor for high carbapenem resistance in CRPA clinical isolates[30,31]. High resistance levels to many antimicrobial agents, including carbapenems, in CRPA isolates from patients with VAP have previously been reported[32-34], indicating multidrug resistance and challenges in treatment. Furthermore, VAP developed relatively late in the study population, with a median symptom onset of 6 days. A late-onset CRPA was defined as CRPA infections occurring ≥5 days after intubation, aligning with established definitions of late-onset VAP. The reasons for this delayed onset are likely due to its association with late-onset

CRPA infections. Key contributing factors of prolonged mechanical ventilation probably include biofilm formation on endotracheal tubes, antibiotic selection pressure favoring resistant strains, and weakened host immunity in critically ill patients.

The MIC evaluation showed that susceptibility to last-line antimicrobial agents for treating CRPA infections, especially carbapenems, was notably poor. Consistent with previous studies[31,35], our CRPA clinical isolates were highly resistant to carbapenems with high MIC values. Similar to the findings of Liu et al. (2023), it was reported that approximately 98% and 87% of CRPA isolates were resistant to imipenem and meropenem, respectively[31]. Most isolates were also resistant to ceftazidime, cefepime, ciprofloxacin, levofloxacin, amikacin, gentamicin, and piperacillin. Moreover, resistance to β-lactam/β-lactamase inhibitors (piperacillin/tazobactam, ticarcillin/clavulanate, and cefoperazone/sulbactam) was found in these isolates. Another study by Asuphon et al. (2017) showed that CRPA isolates from patients admitted to Siriraj Hospital, Bangkok, Thailand, were resistant to imipenem (MIC_{90} of >32 μg/mL), meropenem (MIC_{90} of >32 μg/mL), and doripenem (MIC_{90} of >6 μg/mL)[35], indicating a stable or increasing trend in carbapenem resistance, confirming the overuse or misuse of carbapenems. CRPA clinical isolates commonly resist carbapenems through several mechanisms. These include the production of carbapenemases that break down the β-lactam antibiotics, the overexpression of efflux pumps (e.g., MexAB-OprM) that expel the drug from the bacterial cell, and the loss or modification of outer membrane porins (e.g., OprD) that limit the entry of antibiotics[36,37]. Hence, we hypothesize that there may be an association between carbapenem MIC levels and carbapenem resistance mechanisms. The combination of these mechanisms likely contributes to increased carbapenem MIC levels in CRPA clinical isolates. For the susceptibility of colistin and fosfomycin, in contrast with previous studies[35,38], most of our CRPA isolates were slightly resistant to colistin and susceptible to fosfomycin. Di *et al.* (2015) revealed that most CRPA isolates were susceptible to colistin, with an MIC_{50} of one μg/mL and an MIC_{90} of 2 μg/mL[22]. Similar results were found in the study by Hao et al. (2020), where the MIC_{50} was ≤0.5 μg/mL[38]. Asuphon et al. (2017) reported that most of the CRPA isolates obtained from another part of Thailand were resistant to fosfomycin, with an MIC_{90} of >1024 μg/mL[35]. However, a previous report from Di *et al.* (2015) showed that the MIC_{50} and MIC_{90} of fosfomycin against CRPA isolates were 64 μg/mL and 256 μg/mL, respectively[22]. This may indicate a shift in resistance patterns, possibly due to increased colistin or decreased fosfomycin use for the treatment of CRPA infection in Thailand or environmental factors that have accelerated resistance development. Furthermore, AMR in CRPA clinical isolates may arise from changes in penicillin-binding proteins that reduce drug binding affinity, as well as from biofilm formation, which further protects CRPA from antibiotic penetration[39,40]. These mechanisms often act together, complicating the treatment of CRPA infections. This further emphasizes the importance of antimicrobial stewardship in reducing the spread of CRPA clinical isolates.

The combination of fosfomycin and colistin revealed that an additive effect (93%) was mostly observed against our CRPA clinical isolates. A synergistic effect of this combination was observed against the 2 isolates, with no antagonism. The time-kill kinetics showed that, in addition to the bactericidal activity of each agent, the synergistic effect of their combination was confirmed against 2 isolates, with a 99% to 99.99% reduction. Di et al. (2015) demonstrated that the synergistic and additive effects of fosfomycin and colistin were observed in approximately 22% and 28% of CRPA isolates, respectively, whereas an antagonistic effect was not observed[22]. The percentage of synergistic

effects in the previous study was much higher than in our study, where it was only 5%. The possible reasons for this discrepancy may include differences in bacterial strains, such as variations in the specimens used in the previous study, as well as differences in genetic backgrounds or resistance mechanisms, which may affect the efficacy of the fosfomycin–colistin combination. The time–kill kinetics in their study demonstrated the synergistic bactericidal activity of this combination against most CRPA isolates within 12 hours. The findings of our study, as well as a previous study, showed that, as monotherapies, both fosfomycin and colistin were bactericidal against CRPA clinical isolates within 2 to 8 hours compared with the growth of the control. Notably, regrowth occurred with both agents after 2 to 8 hours, whereas no regrowth was observed with the combination at an effective concentration. Thus, a combination of fosfomycin and colistin might be considered a therapeutic option for CRPA infection; however, its use should be restricted to specific, high–risk cases in order to minimize unnecessary side effects, prevent resistance development, and avoid the inappropriate use of last–resort antibiotics.

Although our findings revealed the clinical characteristics of patients with VAP caused by CRPA and demonstrated the synergistic efficacy of fosfomycin plus colistin against this pathogen, several limitations should be considered. First, the study lacks genetic characterization of AMR mechanisms in the CRPA clinical isolates. While we focused on the phenotypic evaluation of AMR, further research involving genetic analyses, such as whole–genome sequencing or classical bacterial genetic techniques, would be valuable for uncovering the molecular mechanisms underlying the observed resistance. Second, future studies would benefit from incorporating an in–depth investigation of phenotypic resistance mechanisms, such as carbapenemase production, efflux pump activity, and porin loss, which would provide a more comprehensive understanding of the data and their implications. Third, the potential mechanisms of action for the fosfomycin and colistin combination should be further investigated using transcriptomic and/or proteomic analyses to deepen insights into their synergistic effects. Finally, while the *in vitro* results are promising, further in *vivo* experiments are necessary to fully evaluate the clinical applicability and safety of the fosfomycin–colistin combination, especially in high–risk CRPA infections.

Conclusion

This study demonstrates the clinical characteristics of patients with clinical CRPA–induced VAP and the synergistic activity of fosfomycin and colistin. While prior carbapenem exposure is a potential contributing factor for the observed high carbapenem resistance in these isolates, this study cannot establish a definitive causal relationship. Further research, particularly analytical studies, would be required to confirm the role of prior carbapenem exposure in the development of resistance. Our findings also revealed that many CRPA isolates were resistant to colistin, classified as carbapenem– and colistin–resistant isolates. Synergy testing showed that combining fosfomycin and colistin was synergistic against a few CRPA isolates, whereas this combination was additive against most isolates. Therefore, we recommend targeting this antibiotic combination in niche cases, not as a generic antibiotic therapy.

Author contributions

A.C. was responsible for conducting the experiments with the assistance of O.P., R.S., S.C. (Sanicha Chumtong), and P.C., and writing the original manuscript draft. S.C. (Sarunyou Chusri), R.P., P.S., N.K., S.K., and S.C. (Sorawit Chittrakarn) were responsible for conceptualization, validation, formal analysis, investigation, resources, data curation, writing, reviewing, editing, and supervision. A.C.,

O.P., and S.C. (Sarunyou Chusri) were responsible for funding acquisition. All authors have read and agreed to the published version of the manuscript.

Ethical statement

The study was conducted following the Declaration of Helsinki and approved by the Human Research Ethics Committee (HREC) of the Prince of Songkla University (protocol code: 64-284-14-1).

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Conflicts of interest

All authors have no conflicts of interest to declare.

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