Journal of Microbiology, Immunology and Infection (2019) 52, 273–281

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

Original Article

Colistin-sparing regimens against *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates: Combination of tigecycline or doxycycline and gentamicin or amikacin

Hung-Jen Tang [a,b], Chih-Cheng Lai [c], Chi-Chung Chen [d,e], Chun-Cheng Zhang [a], Tzu-Chieh Weng [a], Yu-Hsin Chiu [f], Han-Siong Toh [a], Shyh-Ren Chiang [a], Wen-Liang Yu [d], Wen-Chien Ko [g,*], Yin-Ching Chuang [a,d,f,*]

[a] *Department of Medicine, Chi Mei Medical Center, Tainan, Taiwan*
[b] *Department of Health and Nutrition, Chia Nan University of Pharmacy and Science, Tainan, Taiwan*
[c] *Department of Intensive Care Medicine, Chi Mei Medical Center, Liou Ying, Tainan, Taiwan*
[d] *Department of Medical Research, Chi Mei Medical Center, Tainan, Taiwan*
[e] *Institute of Biotechnology, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan*
[f] *Department of Medicine, Chi Mei Medical Center, Liou Ying, Tainan, Taiwan*
[g] *Department of Medicine, National Cheng Kung University Medical College and Hospital, Tainan, Taiwan*

Received 5 August 2015; received in revised form 4 January 2016; accepted 9 March 2016
Available online 31 March 2016

KEYWORDS
aminoglycoside;
doxycycline;
KPC;
synergism;
tigecycline

Abstract *Background/Purpose: In vitro* studies of the combination of an aminoglycoside with tigecycline or doxycycline against *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* isolates are rarely published. The goal of this study was to evaluate the antibacterial activity of the combination regimens.
Methods: Thirteen genetically different KPC-producing *K. pneumoniae* isolates were randomly selected. Drug concentrations of amikacin, gentamicin, tigecycline, and doxycycline were adjusted to 1-, 1/2-, and 1/4-fold of respective minimum inhibitory concentrations (MICs).

* Corresponding authors. Yin-Ching Chuang, Department of Medical Research, Chi Mei Medical Center, Number 901, Chung-Hwa Road, Yung-Kang City, Tainan, 710, Taiwan. Wen-Chien Ko, Department of Internal Medicine, National Cheng Kung University Hospital, Number 138, Sheng Li Road, 704, Tainan, Taiwan.
E-mail addresses: winston3415@gmail.com (W.-C. Ko), chuangkenneth@hotmail.com (Y.-C. Chuang).

http://dx.doi.org/10.1016/j.jmii.2016.03.003

Each drug alone or the combinations of amikacin or gentamicin with tigecycline or doxycycline were tested by combination studies.
Results: Treatment with the 1× MIC concentration in combinations of amikacin or gentamicin and tigecycline or doxycycline for 24 hours resulted in bactericidal activity of 84—100% in the isolates. Treatment with 1/2× MIC combinations resulted in synergism of 69—100% in the isolates. Notably, doxycycline plus gentamicin or amikacin was synergistic for all tested isolates. However, bactericidal or synergistic effect was barely evident following 1/4× MIC combinations. There was no antagonism in any of the combination regimens.
Conclusion: Enhanced activity was noted following treatment with doxycycline combined with gentamicin or amikacin against KPC-producing *K. pneumoniae* isolates, warranting further *in vitro* and animal investigations before clinical application.

Introduction

Global spread of carbapenem-resistant *Enterobacteriaceae* (CRE) is a major health problem and a clinical challenge for physicians.[1,2] Additional carbapenemases, including *Klebsiella pneumoniae* carbapenemases (KPCs), emerged in Gram-negative bacteria due to extensive use of carbapenems.[3] A national surveillance of CRE in Taiwan in 2011 found a clonal dissemination of KPC-2-producing isolates during the time period, and the prevalence rate of KPC-producing *K. pneumoniae* was 22.3% in Taiwan in 2012.[4—6]

The optimal treatment for infections due to carbapenemase-producing organisms is uncertain.[7] Selection of antibiotic therapy should be tailored to antimicrobial susceptibility results for agents outside the beta-lactam and carbapenem classes, such as colistin, tigecycline, or fosfomycin.[8—10] A recent study of the therapeutic efficacy of various regimens for bloodstream infections caused by KPC-producing *K. pneumoniae* emphasized the importance of combination therapy.[11] Doxycycline was found to be active *in vitro* against some KPC isolates, which was considered as a component of combination therapy.[12] Additionally, doxycycline is almost completely absorbed, with a bioavailability of $>$ 80% (average, 95%), an average area under the curve for daily 200 mg intravenous (IV) doses from 61 mg·h/L to 112 mg·h/L, and a C_{max} of 200 mg from 4.8 mg/L to 6.5 mg/L. Doxycycline also exhibited excellent hepatic/biliary penetration and acceptable concentrations in epithelial-lining fluid.[13] Moreover, Elemam et al[14] demonstrated that rifampin, doxycycline, or tigecycline in combination with polymyxin B exhibited synergistic *in vitro* activity against KPC-producing *K. pneumoniae* isolates. Amikacin was also shown to be effective for treatment against Gram-negative bacteria isolated from respiratory tracts, with peak serum concentration ranges of ~17—26 μg/mL after a 7.5-mg/kg IV drip administration.[15—17] Furthermore, amikacin plus doripenem was reported to be an effective combination therapy in both *in vitro* and *in vivo* infection models involving KPC-producing *K. pneumoniae* isolates.[18]

Tigecycline plus gentamicin or colistin was effective in treating KPC-producing *K. pneumoniae* infections in trauma intensive care unit patients without other comorbidities.[19] Additionally, 15 articles involving 55 unique cases were reviewed, showing that tigecycline and aminoglycosides were associated with favorable outcomes in the majority of KPC-producing *K. pneumoniae* infections.[20] However, *in vitro* studies of the combination effect of an aminoglycoside and tigecycline or doxycycline have been rarely reported. The goal of the study was to evaluate the *in vitro* antibacterial activity of the combinations of an aminoglycoside (gentamicin or amikacin) and tigecycline or doxycycline against KPC-producing *K. pneumoniae* isolates.

Methods

Bacterial strains

Thirty-six clinical carbapenem-resistant *K. pneumoniae* isolates were collected between January 2010 and December 2012 from 17 hospitals in a multicenter surveillance in Taiwan. The isolates were stored at −80°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, UK) before use. Carbapenem resistance is defined as a MIC of at least 4 μg/mL for imipenem or meropenem. Species confirmation was performed by standard biochemical methods using a VITEK 2 automated system (bioMérieux, Marcy l'Etoile, France). The carriage of KPC was confirmed by polymerase chain reaction.[21]

In vitro susceptibility

Standard powders of amikacin, ciprofloxacin, doxycycline, ertapenem, gentamicin, levofloxacin, and imipenem were obtained from U.S. Pharmacopeia (Rockville, MD, USA), and meropenem was obtained from Sigma-Aldrich (St. Louis, MO, USA). Doripenem was kindly provided by Shionogi (Osaka, Japan), fosfomycin by Ercros (Barcelona, Spain), and tigecycline by Pfizer (New York, NY, USA). MIC determinations and susceptibility interpretation criteria followed the Clinical Laboratory and Standard Institute (CLSI) and Federal Drug Administration (FDA) standards.[22,23] MICs of the drugs, except tigecycline, were measured by agar dilution in Mueller—Hinton agar (Oxoid, Basingstoke, UK), according to CLSI recommendations. For fosfomycin

susceptibility, glucose-6-phosphate (25 μg/mL) was added to the agar plate. Tigecycline MICs were determined by broth microdilution in freshly prepared Mueller–Hinton broth with 25 μg/mL of calcium and 12.5 μg/mL of magnesium (CAMHB) as recommended by CLSI guidelines.[22,24] *Escherichia coli* ATCC 25922 was included as the control strain in each run of MIC measurements.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed for the KPC *K. pneumoniae* isolates. Briefly, bacterial chromosomal DNAs were digested using *Xba*I (New England Biolabs, Beverly, MA, USA). Electrophoresis was carried out for 22 hours at 14 uC, with pulse times ranging from 2 seconds to 40 seconds at 6 V/cm, using a Bio-Rad CHEF MAPPER apparatus (Bio-Rad Laboratories, Richmond, CA, USA). A dendrogram based on the unweighted pair group was generated using methods previously described.[25] Isolates that had $>$ 80% similarity on the PFGE profiles were considered closely related strains.

Time-killing method

Thirteen KPC-producing isolates were randomly selected for the following study. The *in vitro* determination of inhibitory effects of combination regimens followed the methodology defined by the CLSI.[26] Briefly, bacterial suspensions were diluted to concentrations of 5.0×10^5 colony-forming units (CFU)/mL in fresh Mueller–Hinton broth. Drug concentrations of amikacin, gentamicin, tigecycline, and doxycycline were adjusted to those of 1× MIC, 1/2× MIC, and 1/4× MIC. Each drug alone or the combinations of amikacin or gentamicin and tigecycline or doxycycline were tested. Bacterial counts were measured at 24 hours by enumerating the colonies in 10-fold serially diluted specimens of 100 μL aliquots plated on nutrient agar (Difco Laboratories, Sparks, MD, USA) at 37°C.

Definitions

Synergism or antagonism was defined as a minimum 100-fold reduction or increase of bacterial loads between the combination and the most active constituent after 24 hours. Bacteriostatic activities were defined as the presence of ≥ 2 $\log_{10}$, but < 3 $\log_{10}$ reductions, and bactericidal activities as the presence of ≥ 3 $\log_{10}$ reductions in CFU/mL at 24 hours, relative to the initial inoculum.[26] All experiments were performed in duplicate.

Checkerboard method

To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each combination by the broth-microdilution technique as recommended by the CLSI and as previously described.[22,27,28] The following formulas were used to calculate the FIC index: (1) FIC of drug A = MIC of drug A in combination/MIC of drug A alone, (2) FIC of drug B = MIC of drug B in combination/MIC of drug B alone, and (3) FIC index = FIC of drug A + FIC of drug B. Synergy was defined as a FIC index of ≤ 0.5, indifference was defined as a FIC index of > 0.5, but ≤ 4, and antagonism defined as a FIC index of > 4.[29]

Results

Among 36 KPC-producing *K. pneumoniae* isolates, all contained KPC-2, with the 50% and 90% MIC ($MIC_{50/90}$) values of the 11 drugs shown in Table 1. Amikacin was the most active, with a susceptible rate of 94.4%, followed by tigecycline (86.1%), gentamicin (79%), and doxycycline (66.7%). According to the PFGE profile, 36 KPC-producing *K. pneumoniae* isolates can be differentiated into five types, from A to E (Figure 1). For *in vitro* combination experiments, one to three isolates were randomly selected from the five types, with a total of 13 clinical isolates collected from blood, urine, wound, or sputum. The MICs of amikacin, gentamicin, doxycycline, and tigecycline for these 13 KPC-producing *K. pneumoniae* isolates are listed in Table 2.

Combination studies

The *in vitro* activities of amikacin, gentamicin, tigecycline, or doxycycline at drug concentrations of 1× MIC, 1/2× MIC, and 1/4× MIC alone or in combinations are shown in Tables 3 and 4. For two doxycycline-resistant isolates, numbers 29 and 35 (MIC, $>$ 128 μg/mL and 128 μg/mL, respectively), time-kill studies of doxycycline-containing combinations were not tested.

When KPC-producing *K. pneumoniae* isolates at an inoculum of 5×10^5 CFU/mL were incubated with amikacin at the concentration of 1× MIC plus 1× MIC doxycycline or tigecycline as compared with the initial inoculum, the reduction in CFU at 24 hours ranged from 1.17 $\log_{10}$ to 4.2 $\log_{10}$ and from 2.5 $\log_{10}$ to 4.26 $\log_{10}$, respectively, and such combinations exhibited bactericidal effect against 82% (9/11) and 85% (11/13) of the tested isolates, respectively. When the drug concentrations decreased to 1/2× MIC, bacterial load reductions at 24 hours ranged from 1.94 $\log_{10}$ to 4.2 $\log_{10}$ (amikacin plus doxycycline) and 0.39 $\log_{10}$ to 4.08 $\log_{10}$ (amikacin plus tigecycline), and 64% (7/11) and 31% (4/13) of the isolates exhibit bactericidal effect, respectively. Notably, at concentrations of 1/2× MIC amikacin plus 1/2× MIC doxycycline or tigecycline, these two combinations were synergistic against 100% (11/11) and 85% (11/13) of the isolates, respectively.

In the case of gentamicin at a concentration of 1× MIC plus 1× MIC doxycycline or tigecycline, the reduction in CFU at 24 hours ranged from 2.46 $\log_{10}$ to 4.2 $\log_{10}$ or from 3.72 $\log_{10}$ to 4.38 $\log_{10}$, and bactericidal activity was exhibited against 91% (10/11) and 100% (13/13) of the isolates, respectively. At drug concentrations of 1/2× MIC in combination, gentamicin plus doxycycline or tigecycline exhibited bactericidal and synergistic activity against 73% or 38% and 100% or 68% of the isolates, respectively.

With different drug combinations at variable concentrations, bacteriostatic or bactericidal activity and synergy or antagonism are shown in Table 5. The combinations of 1/2× MIC gentamicin or amikacin plus tigecycline were

Table 1 MICs and interpretative breakpoints of 11 drugs for 36 *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates.

Drugs	MIC (μg/mL)			Susceptible (%)	MIC (μg/mL)		
	50%	90%	Range		S	I	R
Amikacin	2	8	1—>128	94.4	≤16	32	≥64
Gentamicin	<0.5	16	<0.5—>128	79.0	≤4	8	≥16
Doxycycline	4	32	4—64	66.7	≤4	8	≥16
Tigecycline	2	4	1—4	86.1	≤2	4	≥8
Fosfomycin	64	>1024	32—>1024	50.0	≤64	128	≥256
Ciprofloxacin	128	>128	64—>128	0	≤1	2	≥4
Levofloxacin	128	256	32—256	0	≤2	4	≥8
Doripenem	>32	>32	4—>32	0	≤1	2	≥4
Ertapenem	>32	>32	4—>32	0	≤0.5	1	≥2
Imipenem	32	>64	16—>64	0	≤1	2	≥4
Meropenem	>64	>64	16—>64	0	≤1	2	≥4

I = intermediate; R = resistant; S = susceptible.

synergistic for 69.2—84.6% of the isolates. By contrast, 1/2× MIC gentamicin or amikacin plus doxycycline was synergistic for all isolates. At drug concentrations of 1/4× MIC, all four combinations exhibited poor antibacterial activity.

The data associated with the checkerboard method are shown in Table 6. The 50% and 90% FICs (FIC_{50}/FIC_{90}) of tigecycline- and doxycycline-based combinations was ~0.56—1.0 and ~0.5—0.75. The combination effects of amikin/tigecycline and gentamicin/tigecycline in the categories of synergy and indifference was 36.4% versus 63.6% and 28.6% versus 71.4% of the isolates, respectively. However, the combination effects of amikin/doxycycline and gentamicin/doxycycline in the categories of synergy and indifference was 48.5% versus 51.5% and 67.6% versus 32.4% of the isolates, respectively. The FIC index of the combination of gentamicin and doxycycline was the lowest in the category of synergy and indifference for 67.6% and 32.4% of the isolates, respectively, which have the highest percentage of synergy and constituted the most effective regimen. There was no antagonistic effects observed among the four combinations. The results of the checkerboard method were compatible with those of the 24-hour time-killing method.

Discussion

Carbapenemase-producing *K. pneumoniae* infections that originate from contaminated endoscopic equipment have been reported and successfully treated using tigecycline plus amikacin.[30] Compared with treatments involving tigecycline or colistin combined with a carbapenem or an aminoglycoside, a worse outcome was noted in patients receiving tigecycline or colistin monotherapy for the treatment of KPC-producing *K. pneumoniae* bacteremia.[31] Therefore, it is reasonable to recommend a combination of tigecycline or colistin combined with an aminoglycoside for serious infections due to KPC producers in Taiwan, as both drugs were active against local KPC producers. Additionally, according to a therapeutic algorithm, an aminoglycoside in combination with a carbapenem was suggested for bloodstream infections caused by carbapenemase-producing *K. pneumoniae* in instances where the carbapenem MIC of the causative isolate was ≤ 4 μg/mL.[32] The above information and our *in vitro* susceptibility data associated with aminoglycoside treatment alone or with aminoglycoside-containing regimens supported their clinical application for infections due to multidrug-resistant pathogens.

Tigecycline is a glycylcycline that exhibits inhibitory activity *in vitro* against many multidrug-resistant Gram-negative organisms, including KPC-producing *K. pneumoniae* isolates. However, current tigecycline dosages recommended for adults attain low serum concentrations for the treatment of bloodstream infections due to so-called "tigecycline-susceptible" isolates. Sporadic reports of treatment failure were published with regard to tigecycline monotherapy for the treatment of serious multidrug-resistant infections.[33] Moreover, there were increasing numbers of tigecycline-resistant *K. pneumoniae* isolates. The above concerns precluded tigecycline monotherapy, but the efficacy of tigecycline-based combinations was not excluded. Tigecycline in combination with polymyxin B exhibited synergistic activity.[14] Although our 12 KPC-producing *K. pneumoniae* isolates were susceptible to tigecycline, the tigecycline MIC of 10 isolates was 2 μg/mL, and the serum-attainable concentrations of tigecycline were 0.38 μg/mL and 0.93 μg/mL after single-dose injections of 50 mg and 100 mg tigecycline, respectively,[34] each < 2 μg/mL. However, if 1/2× MIC of tigecycline (i.e., 1 μg/mL) was used in combination with gentamicin or amikacin, synergism was present for 70—85% of the tested isolates. Therefore, when we prescribe recommended dosages to formulate tigecycline-based combination regimens, the synergistic effect would not be evident due to low serum levels of tigecycline (< 1 μg/mL). However, higher levels of tigecycline can be achieved in the skin, soft tissues, or lungs, and may overcome such setbacks.

Other agents exhibiting *in vitro* activity against KPC-producing isolates included aminoglycosides, including gentamicin and amikacin, in this study. Moreover, either aminoglycoside in combination with doxycycline at sub-inhibitory concentrations demonstrated synergistic activity against all 13 KPC-producing isolates, irrespective of

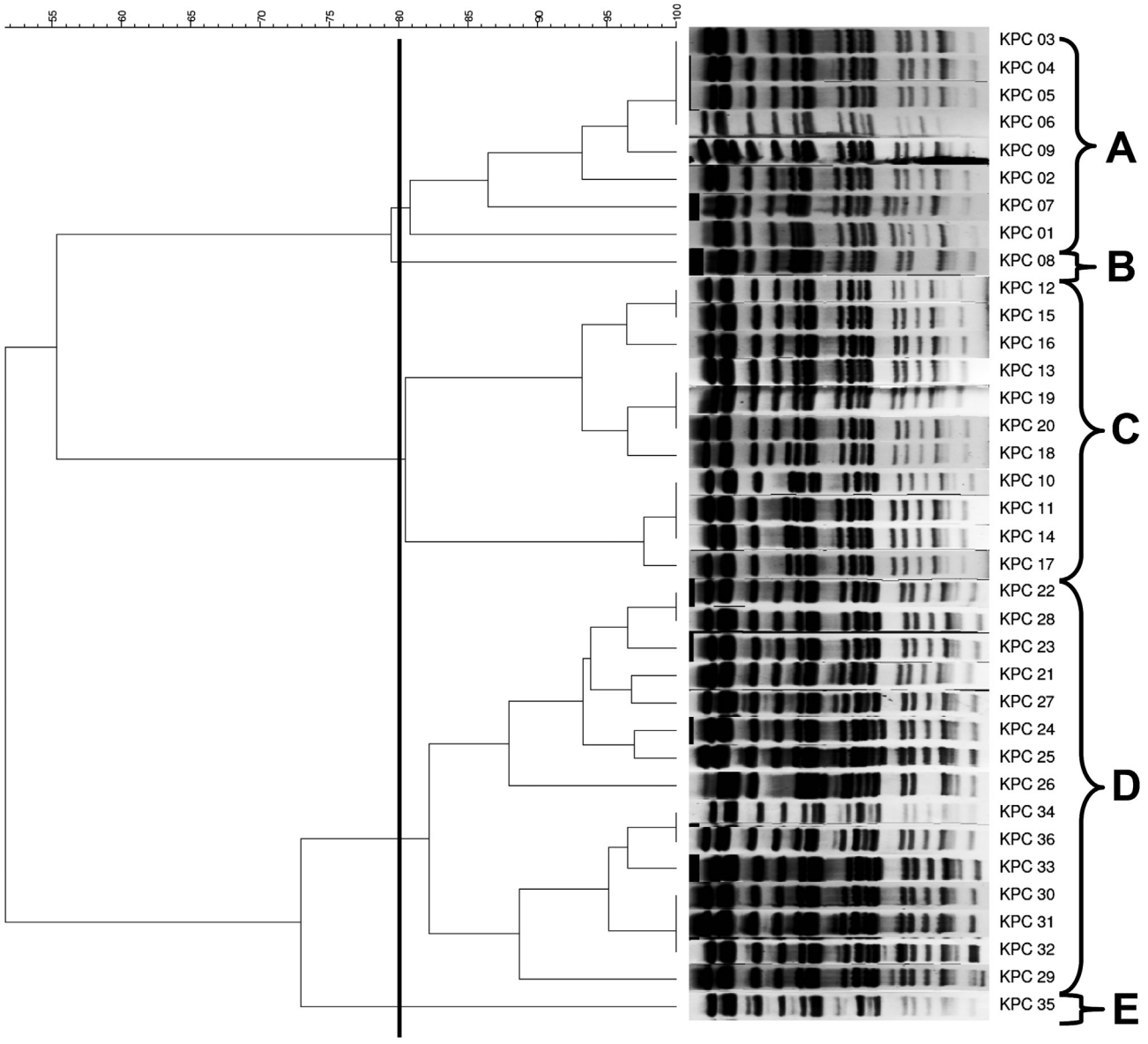

Figure 1. The pulsed-field gel electrophoresis profiles of 36 *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates categorized into five genotypes.

Table 2 Clinical sources, pulsotypes, and MICs of four study drugs of 13 *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates.

Variables	Isolate no.												
	MIC (μg/mL)												
	1	3	7	8	11	12	14	19	22	26	29	32	35
Source	Blood	Urine	Urine	Sputum	BAL	Wound	Sputum	Blood	Urine	Wound	Urine	Sputum	Urine
PFGE type	A6	A1	A5	B	C5	C1	C5	C3	D1	D7	D11	D10	E
Amikacin	32	2	2	32	2	2	1	2	1	2	4	1	4
Gentamicin	32	1	1	32	1	0.5	1	0.5	0.5	1	1	0.5	1
Doxycycline	4	4	4	4	8	4	4	4	4	4	64	4	64
Tigecycline	2	1	1	2	2	2	2	2	2	2	2	2	4

BAL = bronchial alveolar lavage; PFGE = pulsed-field gel electrophoresis.

Table 3 The *in vitro* antibacterial activity of amikacin combined with doxycycline or tigecycline against *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates.

Combination regimens[a]	Colony changes (log_{10} CFU/mL) at 24 h													
	1	3	7	8	11	12	14	19	22	26	29	32	35	Mean
1×AMK + 1×DOX														
vs. initial inoculum	−2.65	−3.83	−4.20	−1.17	−3.81	−3.78	−3.86	−4.08	−3.49	−3.51	ND	−4.20	ND	−3.51
vs. most active drug	−4.81	−1.87	−2.79	−2.97	−4.26	−1.98	−6.92	−4.70	−5.52	−5.38	ND	−6.53	ND	−4.34
1/2×AMK + 1/2×DOX														
vs. initial inoculum	−2.09	−3.83	−4.20	−1.94	−3.51	−3.78	−2.71	−2.40	−3.79	−3.51	ND	−3.00	ND	−3.16
vs. most active drug	−5.30	−6.82	−7.00	−5.30	−6.67	−6.86	−3.00	−5.16	−6.89	−6.76	ND	−5.80	ND	−5.96
1/4×AMK + 1/4×DOX														
vs. initial inoculum	+1.68	−2.93	−2.79	+2.94	−1.27	−1.40	+3.14	+2.71	+3.00	+1.49	ND	+2.35	ND	+0.81
vs. most active drug	−1.52	−5.88	−5.59	−0.42	−4.47	−4.53	0.00	−0.21	−0.21	−1.38	ND	−0.44	ND	−2.24
1×AMK + 1×TGC														
vs. initial inoculum	−3.48	−4.15	−4.26	−2.50	−3.85	−3.72	−3.83	−4.08	−3.79	−4.00	−2.59	−3.90	−3.51	−3.66
vs. most active drug	−0.48	−1.78	−2.62	−4.00	−3.41	−1.26	−7.00	−2.73	−7.00	−5.70	−5.70	−6.48	−1.26	−3.80
1/2×AMK + 1/2×TGC														
vs. initial inoculum	−2.63	+0.76	−0.39	−2.46	+0.84	−3.72	+2.85	−4.08	−0.49	−4.00	+2.99	−3.90	−1.90	−1.24
vs. most active drug	−5.85	−2.02	−3.10	−5.25	−2.32	−6.93	−0.32	−6.85	−3.59	−6.72	−0.12	−7.00	−5.10	−4.24
1/4×AMK + 1/4×TGC														
vs. initial inoculum	+2.94	+2.36	+2.44	+2.56	+2.80	+3.05	+2.77	+2.43	+2.81	+3.00	+3.11	+2.51	+2.89	+2.74
vs. most active drug	−0.28	−0.21	−0.15	−0.16	−0.36	−0.13	−0.40	−0.49	−0.40	0.24	0.00	−0.12	−0.30	−0.21

[a] MIC-equivalent doses in combination.
AMK = amikacin; CFU = colony forming unit; DOX = doxycycline; MIC = minimum inhibitory concentration; ND = not determined; TGC = tigecycline.

Table 4 The *in vitro* antibacterial activity of gentamicin combined with doxycycline or tigecycline against *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates.

Combination regimens[a]	Colony changes (log_{10} CFU/mL) at 24 h													
	1	3	7	8	11	12	14	19	22	26	29	32	35	Mean
1×GM + 1×DOX														
vs. initial inoculum	−3.72	−3.87	−3.87	−2.46	−3.90	−3.76	−3.95	−3.75	−3.85	−3.83	ND	−4.20	ND	−3.74
vs. most active drug	0.00	0.00	−2.56	−3.05	0.00	−6.56	−4.78	−6.72	−4.38	−3.64	ND	0.00	ND	−2.88
1/2×GM + 1/2×DOX														
vs. initial inoculum	−1.24	−3.87	−3.87	−2.24	−3.90	−3.76	−3.95	−1.97	−3.85	−3.83	ND	−4.20	ND	−3.34
vs. most active drug	−4.29	−6.83	−6.87	−4.17	−6.79	−6.83	−5.72	−4.03	−6.85	−5.53	ND	−7.00	ND	−5.90
1/4×GM + 1/4×DOX														
vs. initial inoculum	+2.15	−2.09	−2.49	+2.86	−2.90	+2.14	+2.79	+3.15	+2.23	+0.13	ND	1.14	ND	+0.83
vs. most active drug	−1.13	−4.00	−5.26	−0.42	−3.89	−1.10	1.02	−0.10	−0.77	−3.04	ND	−1.66	ND	−1.85
1×GM + 1×TGC														
vs. initial inoculum	−3.73	−3.75	−3.73	−3.72	−3.76	−3.76	−3.85	−3.82	−3.78	−3.82	−4.38	−4.08	−4.15	−3.87
vs. most active drug	−1.20	0.00	−3.82	−1.81	−5.20	−6.62	−3.73	−5.73	−5.89	−1.08	−3.70	−3.76	0.00	−3.27
1/2×GM + 1/2×TGC														
vs. initial inoculum	−3.43	+2.85	+3.27	−1.27	−1.42	+0.86	−2.20	−2.10	+2.97	−3.82	−4.38	−4.08	−4.15	−1.30
vs. most active drug	−5.64	−0.11	0.00	−2.32	−4.19	−1.72	−5.15	−3.43	−0.25	−6.20	−7.00	−7.00	−7.00	−3.85
1/4×GM + 1/4×TGC														
vs. initial inoculum	+3.15	+2.81	+2.80	+3.13	+2.04	+1.74	+2.30	+1.06	+2.88	+0.93	+2.62	+2.40	+1.48	+2.26
vs. most active drug	−0.12	0.08	−0.11	0.37	−0.05	−1.12	−0.43	−1.96	−0.04	−2.25	0.00	−0.52	−1.38	−0.58

[a] MIC-equivalent doses in combination.
DOX = doxycycline; CFU = colony forming unit; GM = gentamicin; MIC = minimum inhibitory concentration; ND = not determined; TGC = tigecycline.

Table 5 Summary of *in vitro* antibacterial activity of amikacin or gentamicin combined with doxycycline or tigecycline against *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates.

Combination regimens	Total isolate No.	Isolate no. (%)			
		Bacteriostatic	Bactericidal	Synergy	Antagonism
Amikacin + Doxycycline					
1×MIC + 1×MIC	11	1 (9.1)	9 (81.8)	9 (81.8)	0 (0)
1/2×MIC + 1/2×MIC	11	3 (27.3)	7 (63.3)	11 (100)	0 (0)
1/4×MIC + 1/4×MIC	11	2 (18.2)	0 (0)	4 (36.4)	0 (0)
Amikacin + Tigecycline					
1×MIC + 1×MIC	13	2 (15.4)	11 (84.6)	9 (69.2)	0 (0)
1/2×MIC + 1/2×MIC	13	2 (15.4)	4 (30.8)	11 (84.6)	0 (0)
1/4×MIC + 1/4×MIC	13	0 (0)	0 (0)	0 (0)	0 (0)
Gentamicin + Doxycycline					
1×MIC + 1×MIC	11	1 (9.1)	10 (90.9)	7 (63.6)	0 (0)
1/2×MIC + 1/2×MIC	11	1 (9.1)	8 (72.7)	11 (100)	0 (0)
1/4×MIC + 1/4×MIC	11	3 (27.3)	0 (0)	4 (36.4)	0 (0)
Gentamicin + Tigecycline					
1×MIC + 1×MIC	13	0 (0)	13 (100)	8 (61.5)	0 (0)
1/2×MIC + 1/2×MIC	13	2 (15.4)	5 (38.5)	9 (69.2)	0 (0)
1/4×MIC + 1/4×MIC	13	0 (0)	0 (0)	1 (7.7)	0 (0)

MIC = minimal inhibitory concentration.

Table 6 Summary of checkerboard assays of amikacin or gentamicin combined with doxycycline or tigecycline against 13 *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* isolates.

Combinations	Fractional inhibitory concentration index				Synergy (%)	Indifference (%)	Antagonism (%)
	Mean ± SD	Range	50%	90%			
AMK+TGC	0.63 ± 0.19	0.37—1	0.56	1	36.4	63.6	0.0
AMK+DOX	0.56 ± 0.16	0.31—1	0.51	0.75	48.5	51.5	0.0
GM+TGC	0.68 ± 0.19	0.37—1	0.62	1	28.6	71.4	0.0
GM+DOX	0.51 ± 0.20	0.25—1	0.5	0.75	67.6	32.4	0.0

AMK = amikacin; DOX = doxycycline; GM = gentamicin; SD = standard deviation; TGC = tigecycline.

doxycycline or aminoglycoside susceptibility. Such combination regimens at drug concentrations of 1× MIC can be bactericidal to 80—90% of these isolates, indicative of the potential role of these colistin-sparing combinations for KPC producers.

Doxycycline is well established for use as a monotherapy against Gram-negative organisms, including *Klebsiella* species.[35,36] However, clinical efficacy of doxycycline therapy for infections due to multidrug-resistant Gram-negative bacilli has not yet been defined.[14] Although most isolates are susceptible to doxycycline, it is important to note that MIC_{50} values for doxycycline are near the susceptibility breakpoint (4 μg/mL). Moreover, drug concentrations at sites of infection should be taken into account before their clinical use.[20] However, tissue penetration of doxycycline is excellent, and drug levels in most organs and tissues, including kidney, lung, and sinus secretions, are within the therapeutic range.[37] The serum-attainable concentration of doxycycline was 3.5 μg/mL after oral administration of 100 mg doxycycline.[38] For combination therapy using 1/2× MIC of doxycycline (2 μg/mL, which is achievable in serum), synergism is predominant and suggestive of the potential role of doxycycline-based combinations in clinical practice. Jernigan et al[12] suggested that doxycycline plus gentamicin was synergistic against 25% of 12 KPC-producing isolates, and they reserved doxycycline-containing regimens for the treatment of uncomplicated cystitis at their center.[12] By contrast, our study found that sub-inhibitory concentrations of an aminoglycoside combined with doxycycline exhibited synergistic activity against all tested isolates. Thus, doxycycline alone or in combination with an aminoglycoside possesses potential antibacterial activity and can be considered an alternative for CRE infections.

In conclusion, enhanced antibacterial activity of doxycycline plus gentamicin or amikacin was observed against KPC-producing *K. pneumoniae* isolates, and warrants clinical validation of their therapeutic efficacy.

Conflicts of interest

None to declare.

Acknowledgments

We thank Zih-Ting Chen and Yan-Chang Yang and the staff in the Research Laboratory of Infectious Diseases at the Chi-Mei Medical Center for their invaluable assistance with laboratory experiments. This study was supported by grants from the National Science Council of Taiwan (NSC-102-2314-13-384-009-MY2) and Chi-Mei Foundation Hospital, Taiwan (CMFHR10360).

References

1. Liu SW, Chang HJ, Chia JH, Kuo AJ, Wu TL, Lee MH. Outcomes and characteristics of ertapenem-nonsusceptible Klebsiella pneumoniae bacteremia at a university hospital in Northern Taiwan: a matched case-control study. *J Microbiol Immunol Infect* 2012;**45**:113—9.
2. Nordmann P, Naas T, Poirel L. Global spread of Carbapenemase-producing *Enterobacteriaceae*. *Emerg Infect Dis* 2011;**17**:1791—8.
3. Hung KH, Yan JJ, Lu JJ, Chen HM, Wu JJ. Characterization of the modified Hodge test-positive isolates of *Enterobacteriaceae* in Taiwan. *J Microbiol Immunol Infect* 2013;**46**:35—40.
4. Jean SS, Lee WS, Hsueh PR. Nationwide spread of *Klebsiella pneumoniae* carbapenemase-2-producing K. pneumoniae sequence type 11 in Taiwan. *J Microbiol Immunol Infect* 2013;**46**:317—9.
5. Chiu SK, Wu TL, Chuang YC, Lin JC, Fung CP, Lu PL, et al. National surveillance study on carbapenem non-susceptible *Klebsiella pneumoniae* in Taiwan: the emergence and rapid dissemination of KPC-2 carbapenemase. *PLoS ONE* 2013;**8**:e69428.
6. Chen YT, Lin JC, Fung CP, Lu PL, Chuang YC, Wu TL, et al. KPC-2-encoding plasmids from *Escherichia coli* and *Klebsiella pneumoniae* in Taiwan. *J Antimicrob Chemother* 2014;**69**:628—31.
7. Zavascki AP, Barth AL, Gaspareto PB, Goncalves AL, Moro AL, Fernandes JF, et al. Risk factors for nosocomial infections due to *Pseudomonas aeruginosa* producing metallo-beta-lactamase in two tertiary-care teaching hospitals. *J Antimicrob Chemother* 2006;**58**:882—5.
8. Jean SS, Lee WS, Lam C, Hsu CW, Chen RJ, Hsueh PR. Carbapenemase-producing Gram-negative bacteria: current epidemics, antimicrobial susceptibility and treatment options. *Future Microbiol* 2015;**10**:407—25.
9. Kanj SS, Kanafani ZA. Current concepts in antimicrobial therapy against resistant gram-negative organisms: extended-spectrum beta-lactamase-producing *Enterobacteriaceae*, carbapenem-resistant *Enterobacteriaceae*, and multidrug-resistant Pseudomonas aeruginosa. *Mayo Clin Proc* 2011;**86**:250—9.
10. Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N. What remains against carbapenem-resistant *Enterobacteriaceae*? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. *Int J Antimicrob Agents* 2011;**37**:415—9.
11. Tumbarello M, Viale P, Viscoli C, Trecarichi EM, Tumietto F, Marchese A, et al. Predictors of mortality in bloodstream infections caused by Klebsiella pneumoniae carbapenemase-producing K. pneumoniae: importance of combination therapy. *Clin Infect Dis* 2012;**55**:943—50.
12. Jernigan MG, Press EG, Nguyen MH, Clancy CJ, Shields RK. The combination of doripenem and colistin is bactericidal and synergistic against colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2012;**56**:3395—8.
13. Agwuh KN, MacGowan A. Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. *J Antimicrob Chemother* 2006;**58**:256—65.
14. Elemam A, Rahimian J, Doymaz M. In vitro evaluation of antibiotic synergy for polymyxin B-resistant carbapenemase-producing *Klebsiella pneumoniae*. *J Clin Microbiol* 2010;**48**:3558—62.
15. Farago E, Kiss J, Gomory A, Aranyosi J, Juhasz I, Mihoczy L. Amikacin: in vitro bacteriological studies, levels in human serum, lung and heart tissue, and clinical results. *Int J Clin Pharmacol Biopharm* 1979;**17**:421—8.
16. Tayman C, El-Attug MN, Adams E, Van Schepdael A, Debeer A, Allegaert K, et al. Quantification of amikacin in bronchial epithelial lining fluid in neonates. *Antimicrob Agents Chemother* 2011;**55**:3990—3.
17. Saux MC, Crockett R, Fourtillan JB, Leng B, Couraud L. Diffusion of amikacin in the lungs. *Pathol Biol (Paris)* 1986;**34**:113—7.
18. Hirsch EB, Guo B, Chang KT, Cao H, Ledesma KR, Singh M, et al. Assessment of antimicrobial combinations for *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*. *J Infect Dis* 2013;**207**:786—93.
19. Sbrana F, Malacarne P, Viaggi B, Costanzo S, Leonetti P, Leonildi A, et al. Carbapenem-sparing antibiotic regimens for infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* in intensive care unit. *Clin Infect Dis* 2013;**56**:697—700.
20. Hirsch EB, Tam VH. Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *J Antimicrob Chemother* 2010;**65**:1119—25.
21. Ma L, Siu LK, Lin JC, Wu TL, Fung CP, Wang JT, et al. Updated molecular epidemiology of carbapenem-non-susceptible *Escherichia coli* in Taiwan: first identification of KPC-2 or NDM-1-producing E. coli in Taiwan. *BMC Infect Dis* 2013;**13**:599.
22. Clinical and Laboratory Standards Institute (CLSI). *Performance standards for antimicrobial susceptibility testing: 16th informational supplement. Document M100—S22*. Wayne, PA: CLSI; 2012.
23. Brown SD, Traczewski MM. Comparative in vitro antimicrobial activity of tigecycline, a new glycylcycline compound, in freshly prepared medium and quality control. *J Clin Microbiol* 2007;**45**:2173—9.
24. Clinical and Laboratory Standards Institute (CLSI). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically—8th Edition. Approved Standard M07—A9*. Wayne, PA: CLSI; 2009.
25. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995;**33**:2233—9.
26. National Committee for Clinical Laboratory Standards. *Methods for determining bactericidal activity of antimicrobial agents: approved guideline. Document M26-A*. Wayne, PA: CLSI; 1999.
27. Eliopoulos GM, Moellering Jr RC. Antibiotics in laboratory medicine. In: Lorian V, editor. *Antimicrobial combinations*. 4th ed. Baltimore, MD: The Williams & Wilkins Co.; 1996. p. 330—96.
28. Moody JA. Synergy testing: broth microdilution checkerboard and broth macrodilution methods. In: Eisenberg HD, editor. *Clinical microbiology procedures handbook*. Washington, D.C.: American Society for Microbiology; 1992. 5.18.1—23.

29. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob Agents Chemother* 1996;**40**:1914—8.
30. Alrabaa SF, Nguyen P, Sanderson R, Baluch A, Sandin RL, Kelker D, et al. Early identification and control of carbapenemase-producing Klebsiella pneumoniae, originating from contaminated endoscopic equipment. *Am J Infect Control* 2013;**41**:562—4.
31. Qureshi ZA, Paterson DL, Potoski BA, Kilayko MC, Sandovsky G, Sordillo E, et al. Treatment outcome of bacteremia due to KPC-producing *Klebsiella pneumoniae*: superiority of combination antimicrobial regimens. *Antimicrob Agents Chemother* 2012; **56**:2108—13.
32. Daikos GL, Markogiannakis A, Souli M, Tzouvelekis LS. Bloodstream infections caused by carbapenemase-producing *Klebsiella pneumoniae*: a clinical perspective. *Expert Rev Anti Infect Ther* 2012;**10**:1393—404.
33. Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis* 2009;**9**:228—36.
34. Meagher AK, Ambrose PG, Grasela TH, Ellis-Grosse EJ. Pharmacokinetic/pharmacodynamic profile for tigecycline-a new glycylcycline antimicrobial agent. *Diagn Microbiol Infect Dis* 2005;**52**:165—71.
35. Rodriguez-Hernandez MJ, Pachon J, Pichardo C, Cuberos L, Ibanez-Martinez J, Garcia-Curiel A, et al. Imipenem, doxycycline and amikacin in monotherapy and in combination in *Acinetobacter baumannii* experimental pneumonia. *J Antimicrob Chemother* 2000;**45**:493—501.
36. Goel N, Chaudhary U, Aggarwal R, Bala K. Antibiotic sensitivity pattern of gram negative bacilli isolated from the lower respiratory tract of ventilated patients in the Intensive care unit. *Indian J Crit Care Med* 2009;**13**:148—51.
37. Cunha BA, Sibley CM, Ristuccia AM. Doxycycline. *Ther Drug Monit* 1982;**4**:115—35.
38. Selvadurai M, Meyyanathan SN, Rajan S, Padmanaban G, Suresh B. Determination of doxycycline in human plasma by liquid chromatography-mass spectrometry after liquid-liquid extraction and its application in human pharmacokinetics studies. *J Bioequiv Availab* 2010;**2**:93—7.