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ARTICLE

In vitro activity of minocycline combined with aminoglycosides against *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*

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Abstract

This study assessed the in vitro antibacterial activity of minocycline-aminoglycoside combination against *Klebsiella pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae*. Seventy non-duplicate clinical isolates of KPC-producing *K. pneumoniae* were collected from patients with bloodstream infections. The synergistic activity of minocycline-aminoglycoside combination was studied by the checkerboard method and time-kill assays in strains with different susceptibilities, and the mutant prevention concentration (MPC) and mutant selection window (MSW) of drugs alone and in combination were determined. The checkerboard method found this combination displayed synergistic and partial synergistic activity against aminoglycoside-susceptible isolates, but indifferent activity against aminoglycoside-resistant isolates. Time-kill assays further demonstrated strong synergistic and bactericidal effect of this combination existed against isolates which were susceptible to both drugs. But for resistant isolates, the time-kill assays showed no synergy. The MPCs of minocycline or aminoglycosides were 8- to 32-fold higher than the MICs, suggesting the MSWs of these drugs were quite wide. For the antibiotic combinations, the addition of 1×MIC concentration of amikacin or gentamicin could reduce the MPCs of minocycline by 4- to 16-fold. Generally, no mutants recovered in the plates containing 1×MIC concentration of minocycline and 2×MIC concentration of amikacin or gentamicin. In summary, these results suggest that minocycline-aminoglycoside combination can be an alternative for infections caused by KPC-producing *K. pneumoniae* because this combination displays strong synergistic and bactericidal activity in susceptible isolates, and can effectively prevent the emergence of resistant mutants. Further in vitro pharmacokinetic/pharmacodynamic studies and clinical trials should be performed to fully evaluate the efficacy of this drug combination.

Introduction

Klebsiella pneumoniae carbapenemase (KPC)-producing *K. pneumoniae* has now spread internationally, and become endemic in many countries and regions [1]. Because KPC-producing *K. pneumoniae* usually causes serious infections in debilitated and immunocompromised patients, the treatment should be timely and rapidly efficacious [2]. However, KPC-producing *K. pneumoniae* is not only resistant to β-lactams, but also shows decreased susceptibility to other antimicrobial classes commonly used in clinical practice [2]. The paucity of effective treatment options for this super bug has resulted in prolonged hospital stays and high mortality rates.

By far, only a few antimicrobials, such as polymyxins, tigecycline, some aminoglycosides, and the combination of ceftazidime and avibactam, show favorable in vitro activity against KPC-producing *K. pneumoniae*. However,

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monotherapy with these drugs is not advised because clinical studies have found that the mortality rates for patients on monotherapy are higher than for those receiving combination therapy [3, 4]. Moreover, due to the wide use, resistance has challenged these last-resort treatment options. Thus, combination regimens based on carbapenems, polymyxins, and tigecycline have been widely used for the treatment of KPC-producing *K. pneumoniae* infections in clinical practice [5]. Commonly, minocycline is not an option for the treatment of KPC-producing *K. pneumoniae* since its derivate, tigecycline, has better antimicrobial activity. However, the efficacy of tigecycline in treating bloodstream infections is controversial because of its large volume of distribution and its low concentration in the blood [6]. Minocycline has comparatively good in vitro activity against multidrug-resistant (MDR) Gram-negative bacteria, it shows attractive antimicrobial activity compared to doxycycline, and it has a pharmacokinetic advantage over tigecycline in the blood [7]. Therefore, minocycline was reintroduced onto the US market in 2009, to address the increasing resistance to current first-line agents.

Few studies have investigated the interactions between minocycline and other antibiotics for the treatment of KPC-producing *K. pneumoniae*. Only one recent study has reported that the synergistic activity of a polymyxin B–minocycline combination is dependent on the polymyxin B susceptibility of the strain [8]. Previous studies demonstrated that resistant mutant isolates most likely occurs when antimicrobial concentrations fall in a specific range called the mutant selection window (MSW) [9]. The lower boundary of the MSW is approximate to the minimal inhibitory concentration (MIC), and the upper boundary is the mutant prevention concentration (MPC), which represents a concentration threshold above which no single-step drug-resistant mutant strains can be selected [9]. Therefore, comparison of the MPC and MSW of antibiotics alone and in combination is be useful in telling whether combination therapy can reduce the chance of resistant mutants emerging. The objectives of this study were to evaluate the in vitro synergistic activity of minocycline combined with aminoglycosides against KPC-producing *K. pneumoniae*, and to investigate the ability of this combination to prevent resistance by determining the mutant prevention concentrations (MPCs) of the drugs individually and in combination.

Materials and methods

Bacterial strains and antimicrobial agents

A total of 70 non-duplicate clinical isolates of KPC-producing *K. pneumoniae* were isolated from patients with bloodstream infections in 2 tertiary hospitals in Beijing, China, from June 2014 to December 2016. All isolates were identified with the VITEK® 2 Compact System (bioMérieux, Marcy-l'Étoile, France). Minocycline, amikacin, and gentamicin standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

In vitro susceptibility

We used the agar dilution method to determine the minimum inhibitory concentrations (MICs) for the 3 drugs. The experiment was replicated 3 times in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [10]. Briefly, Mueller Hinton agar (MHA; Difco, Franklin Lakes, NJ, USA) plates containing a series of 2-fold concentration increments of each agent were prepared. Then, ~10^4 colony-forming units (CFU) of bacterial cells were inoculated with an autoclaved replicator and incubated at 37.5 °C for 20 h. The MIC was defined as the lowest drug concentration where no visible colonies grew. *Escherichia coli* ATCC25922 was used as the quality control strain for each batch of tests.

Synergy testing of drug combinations with the checkerboard method

Based on the susceptibility results, 24 amikacin-susceptible isolates, 22 gentamicin-susceptible isolates, and 10 amikacin/gentamicin dual resistant isolates were randomly selected for the synergy testing. The checkerboard method was performed according to our previous study [11]. In brief, the drugs were diluted with cation-adjusted Mueller-Hinton Broth (CA-MHB) (Difco, Franklin Lakes, NJ, USA) into a series of concentrations based on the MICs for the tested isolates. Then, we added 50 µl minocycline, 50 µl amikacin or gentamicin, and 100 µl bacterial suspension (1×10^6 CFU/mL) into 96-well microdilution plates. After their contents were mixed with a vortex mixer, the plates were cultured at 37.5 °C for 20 h.

Synergy was assessed with the fractional inhibitory concentration index (FICI): FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). The FICI value was interpreted as follows: synergism, FICI ≤ 0.5; partial synergism, 0.5 < FICI < 1; additivity, FICI = 1; indifference, 1 < FICI < 4; and antagonism, FICI > 4 [12]. The cumulative inhibition ratio (CIR) was defined as the percentage of isolates that were inhibited at a certain antibiotic concentration.

Time-kill assays

Four isolates that were susceptible to both minocycline and aminoglycosides, 2 isolates that were susceptible to

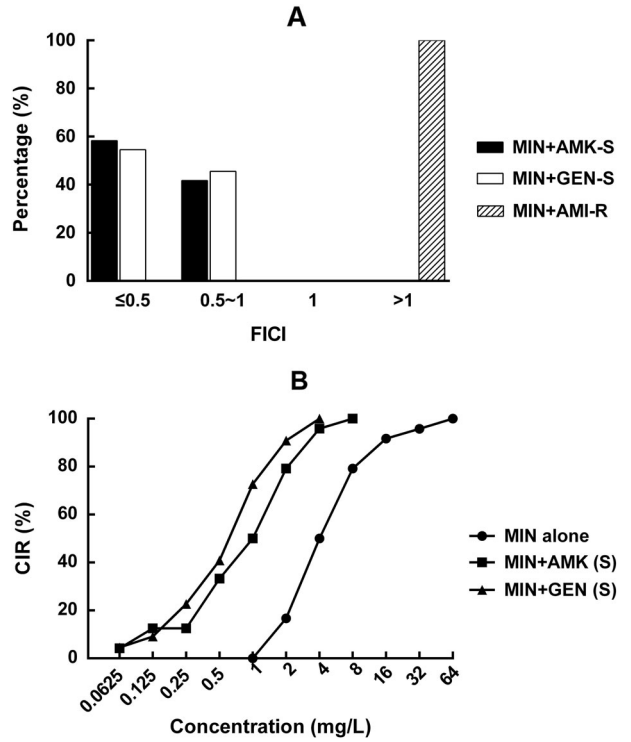

Fig. 1 **a** fractional inhibitory concentration index (FICI) of minocycline (MIN) combined with amikacin (AMK) and gentamicin (GEN) for amikacin-susceptible (AMK-S), gentamicin-susceptible (GEN-S) and aminoglycoside-resistant (AMI-R) KPC-producing *K. pneumoniae*; **b** cumulative inhibition ratio (CIR) of minocycline alone and in combination with amikacin or against aminoglycoside-susceptible (S) KPC-producing *K. pneumoniae*

minocycline and highly resistant to aminoglycosides (MIC ≥ 128 mg/L), and 2 isolates that were highly resistant to minocycline (MIC ≥ 64 mg/L) and susceptible to aminoglycosides were randomly selected for the following study. Briefly, bacterial suspensions were diluted to 1×10^5 CFU/mL with fresh CA-MHB. For isolates with dual susceptibility to minocycline and aminoglycosides, the concentrations of minocycline, amikacin, and gentamicin were adjusted to the 50% of MIC; for isolates susceptible to minocycline and resistant to aminoglycosides, the concentration of minocycline was adjusted to the 50% of MIC, and amikacin and gentamicin were adjusted to their CLSI susceptibility breakpoints (amikacin, 16 mg/L; gentamicin, 4 mg/L); for isolates resistant to minocycline and susceptible to aminoglycosides, the concentration of minocycline was adjusted to its CLSI susceptibility breakpoint (4 mg/L), and amikacin and gentamicin were adjusted to their 50% of MIC. Bacterial counts were measured at 0, 3, 6, 12, and 24 h by enumerating the colonies in 10-fold serially diluted specimens of 100 µl aliquots plated on MHA at 37.5 °C for 20 h. All of the in vitro time-kill experiments were performed in triplicate on different days. The results are expressed as the mean ± standard deviation (SD).

A reduction of ≥ 3 log10 CFU/mL compared to the original inoculum was considered bactericidal. Compared with the most active drug in the pair, a further reduction of ≥ 2 log10 CFU/mL in combination was defined as synergism, a reduction of < 2 log10 CFU/mL was defined as indifference, and an increase of ≥ 2 log10 CFU/mL was defined as antagonism [13].

Determination of MPCs and MSWs

The MPCs of minocycline and the aminoglycosides alone were determined in 10 susceptible isolates, and the MPCs of the combinations were determined in 3 randomly selected isolates. The MPCs were determined with a modified agar dilution method [11]. In brief, ~0.3×1010 CFU/mL bacterial cells were placed onto MHA plates with 2-fold concentration increments of minocycline (0–256 mg/L), and amikacin and gentamicin (0.5–128 mg/L) alone, as well as in combination. Each drug concentration was included on at least 4 plates to ensure that the total cell number in the inoculum was about 1.2×1010. The plates were incubated at 37.5 °C for 72 h. The MPC was defined as the lowest antibiotic concentration that prevented the visible growth of mutant colonies.

Results

In vitro susceptibility

Among the 70 KPC-producing *K. pneumoniae* isolates, the rates of susceptibility, intermediate susceptibility, and resistance to minocycline were 50%, 18.6%, and 31.4%, respectively. The MIC_{50} and MIC_{90} for minocycline were 4 and 16 mg/L, respectively. The susceptibility rates to amikacin and gentamicin were 51.4% and 32.9%, respectively.

Synergistic activity between minocycline and aminoglycosides

Figure 1a shows the synergy results from the checkerboard experiments. For all of the isolates susceptible to aminoglycosides, the minocycline–aminoglycoside combinations displayed synergistic and partial synergistic activity. However, for the isolates resistant to aminoglycosides, the FICIs of the minocycline–aminoglycoside combinations of all isolates ranged from 1 to 2, indicating indifference. The curves for the CIRs of minocycline alone and in combination with aminoglycosides for aminoglycoside-susceptible strains are shown in Fig. 1b. The curves shifted dramatically to the left after the addition of amikacin or gentamicin. The MIC_{50} of minocycline decreased from 4 to 1 mg/L in the presence of amikacin and to 0.5 mg/L in the presence of gentamicin.

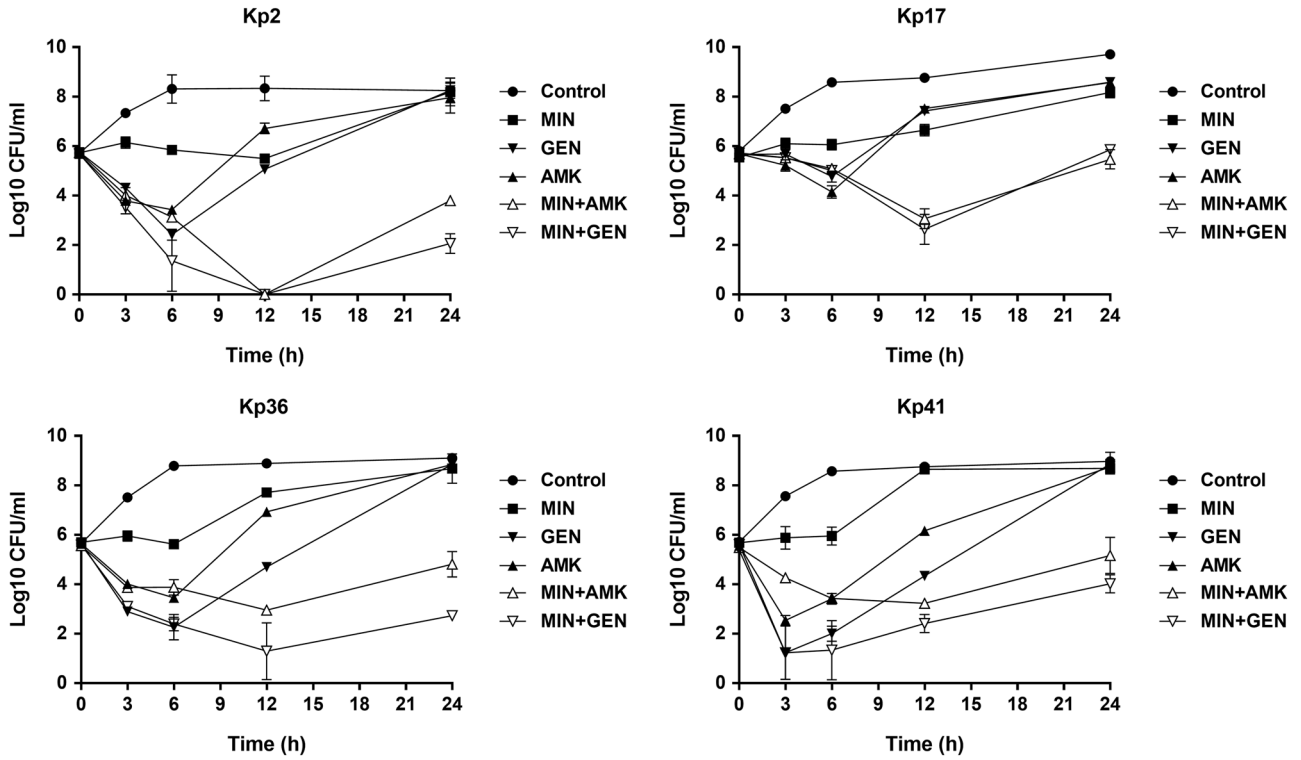

Fig. 2 In vitro time-kill curves of minocycline (MIN), amikacin (AMK), gentamicin (GEN) alone and in combination against KPC-producing *K. pneumoniae*. (Tested strains were all susceptible to both minocycline and aminoglycosides)

Time-kill assays

As shown in Fig. 2, for the dual minocycline/aminoglycoside-susceptible isolates, minocycline alone exhibited bacteriostatic activity, and amikacin or gentamicin alone displayed bactericidal activity in the first 3–6 h; but after then, quick regrowth was observed. Although regrowth was also observed in combination therapy, the rate was much lower than that in monotherapy. At 24 h, synergistic activity was observed in all isolates and bactericidal activity was still observed in 3 isolates. However, for the minocycline-susceptible and highly aminoglycoside-resistant isolates or the highly minocycline-resistant and aminoglycoside-susceptible isolates, the combinations did not display synergistic activity at 24 h (Fig. 3). We did not observe antagonism in this study.

Estimation of the MPCs of drugs singly and in combination

The MICs and MPCs for minocycline and the aminoglycosides alone for the tested isolates are shown in Table 1. The MICs for minocycline ranged from 1 to 4 mg/L, and the MPCs were 8- to 32-fold higher than the MICs. The MICs and MPCs for amikacin were 2- to 8-fold and 4- to 8-fold higher, respectively, than those for gentamicin. For the antibiotic combinations, the addition of 1×MIC concentration of amikacin or gentamicin could reduce the MPCs of minocycline by 4- to 16-fold (Fig. 4). With the exception of the minocycline–amikacin combination in Kp17, no mutants recovered in the plates containing the 1×MIC concentration of minocycline and the 2×MIC concentration of amikacin or gentamicin.

Discussion

Minocycline is the second-generation tetracycline that was first introduced on the market in the 1960s [14]. Although the advent of new β-lactams and fluoroquinolones replaced minocycline for the treatment of systemic infections in the 1980s and 1990s, it has now has become an important option for the treatment of MDR organisms. Previous clinical studies have found that minocycline can be used safely and effectively as a salvage therapy for carbapenem-resistant *Acinetobacter baumannii* infections after patients have failed multiple other antibiotic regimens [15]. We found that most KPC-producing *K. pneumoniae* isolates were susceptible or had intermediate susceptibility to minocycline; the resistance rate of 31.4% suggests its potential for the treatment of infections caused by this organism. However, it is important to note that the MIC_{50} values for

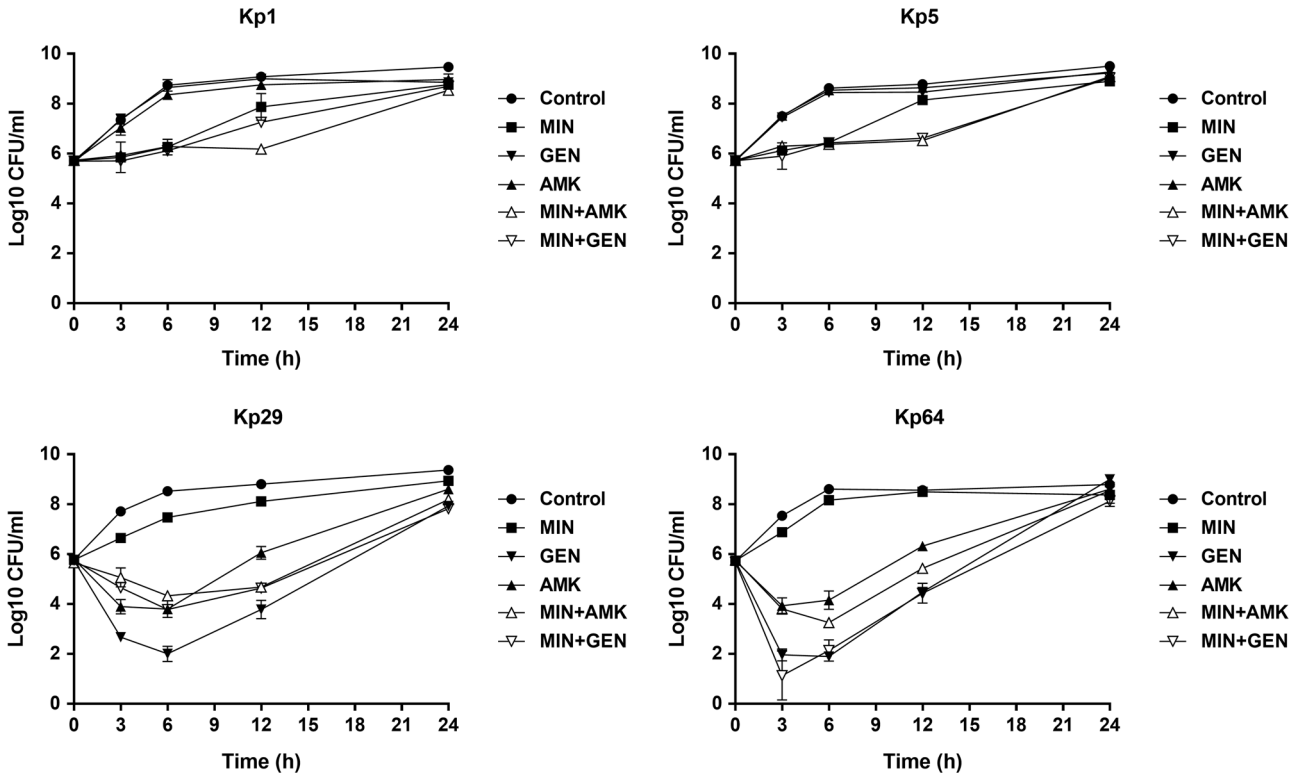

Fig. 3 In vitro time–kill curves of minocycline (MIN), amikacin (AMK), gentamicin (GEN) alone and in combination against KPC-producing *K. pneumoniae*. (Kp1 and Kp5 were susceptible to minocycline and resistant to aminoglycosides; Kp29 and Kp64 were to resistant minocycline and susceptible to aminoglycosides)

Table 1 MICs and MPCs of minocycline, amikacin and gentamicin for 10 KPC-producing *K. pneumoniae* clinical strains

Isolate	Minocycline		Amikacin		Gentamicin	
	MIC	MPC	MIC	MPC	MIC	MPC
Kp2	1	16	1	32	0.125	4
Kp6	4	32	2	64	1	16
Kp7	4	64	2	64	1	8
Kp17	2	32	2	32	0.5	8
Kp25	4	64	1	32	0.25	4
Kp36	2	32	2	64	0.5	8
Kp41	4	64	2	32	1	8
Kp48	1	16	2	64	1	8
Kp59	4	64	2	32	0.5	4
Kp70	4	32	0.5	64	0.25	8

minocycline were near its susceptibility breakpoint (4 mg/L), and the MPC values were 8- to 32-fold higher than the MICs. Therefore, the use of minocycline alone may result in treatment failure and the rapid emergence of resistance when treating KPC-producing *K. pneumoniae* infections. In this study, we evaluated the in vitro synergy between minocycline and aminoglycosides against KPC-producing *K. pneumoniae* with varying susceptibilities to each drug. Using the checkerboard method, we observed synergism and partial synergism in aminoglycoside-sensitive strains, but not in highly aminoglycoside-resistant strains. The time–kill curve assays validated the results of the checkerboard method, and showed that this combination in both minocycline- and aminoglycoside-susceptible isolates could significantly improve bacterial killing.

Previous in vitro and in vivo studies have investigated the effects of minocycline in combination with several antimicrobials against *A. baumannii*, with favorable results. Rodríguez et al. showed that minocycline had bactericidal synergy when combined with rifampicin, colistin, or imipenem in most minocycline-susceptible *A. baumannii* [16]. Yang et al. found that the combination of minocycline and colistin, compared to colistin monotherapy, significantly reduced the number of bacteria in the lungs of mice [17]. A recent study also demonstrated that a polymyxin B–minocycline combination had synergistic activity against KPC-producing *K. pneumoniae*, and that the synergism was most apparent against polymyxin-susceptible isolates [8]. In addition, 2 studies found that tigecycline or doxycycline combined with gentamicin or amikacin synergistically killed MDR-*E. coli* and KPC-producing *K. pneumonia* [18, 19]. And in this study, we noted enhanced activity by

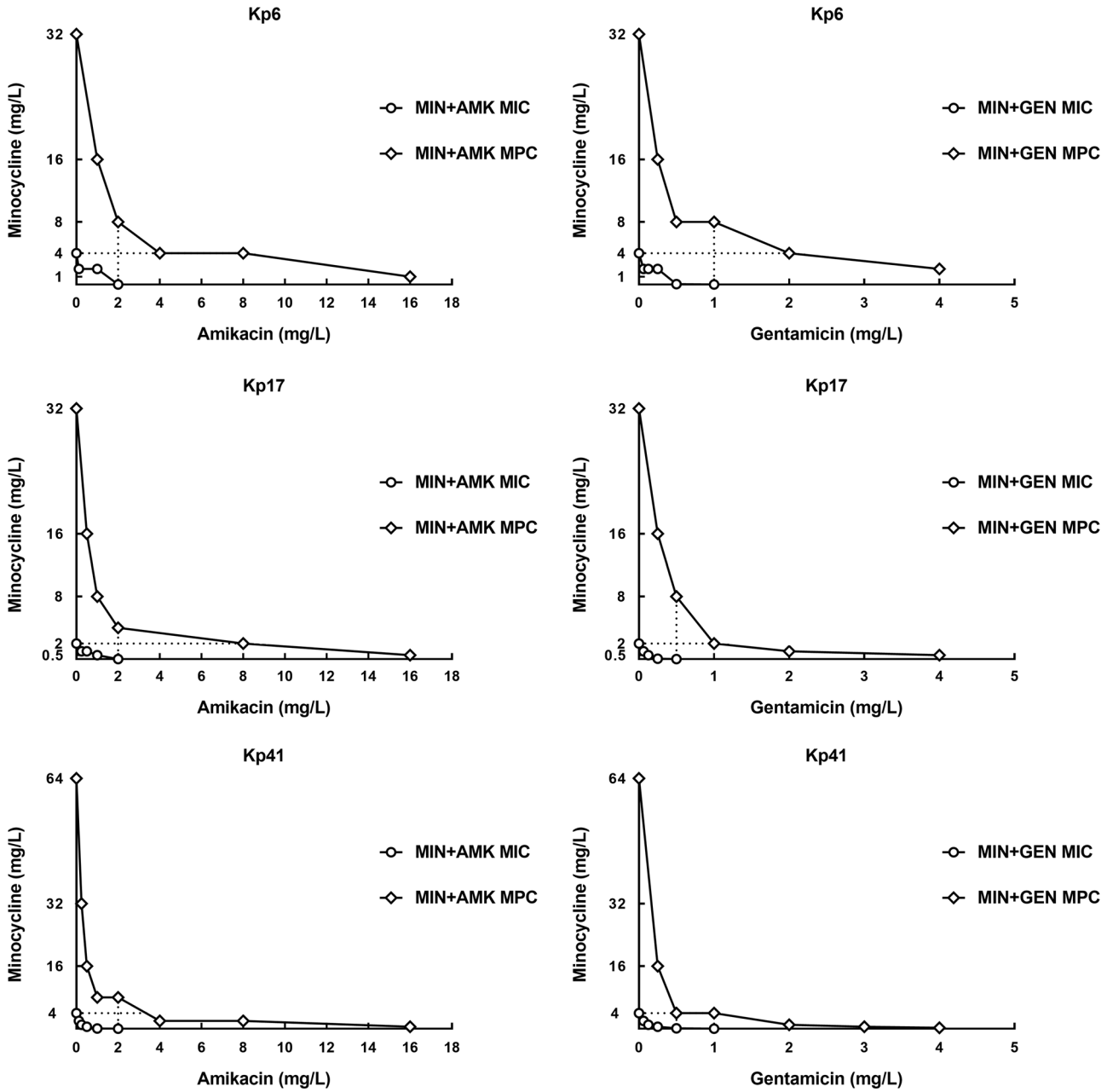

Fig. 4 MPCs and MICs of minocycline (MIN) alone and combined with different concentrations of amikacin (AMK) or gentamicin (GEN) for three KPC-producing *K. pneumoniae* strains

the minocycline–aminoglycoside combination against KPC-producing *K. pneumoniae* isolates. These findings indicated the potential role of combination therapies containing tetracyclines and polymyxins or aminoglycosides in the management of KPC-producing *K. pneumoniae* infections.

In addition to maximizing clinical effectiveness, antimicrobial combination therapy could decrease the potential for resistance emergence because susceptible bacteria have a low probability ($<10^{-10}$) of developing 2 concurrent mutations that allow them to survive treatment with 2 drugs with different antimicrobial mechanisms [20]. We found that the addition of the 1 × MIC concentration of an aminoglycoside could reduce the MPC of minocycline by 4- to 8-fold, and we recovered no resistant mutants after treatment with the 1 × MIC of minocycline (1–4 mg/L) combined with the 2×MIC concentration of amikacin (1–4 mg/L) or gentamicin (0.25–2 mg/L). The mean trough and peak serum concentrations of amikacin were 4.38 mg/L and 23.36 mg/L with a dose of 1000 mg/day [21], and the trough serum concentration of gentamicin in 60% of patients was ≥ 2.5 mg/L with a dose of 8 mg/kg/day [22]. For

minocycline, serum concentrations ranged from 2.1 to 6.6 mg/L with a 200 mg intravenous dose [23]. Therefore, considering the pharmacokinetics of these drugs, a minocycline–aminoglycoside combination may effectively prevent selection for resistance at the current dosage.

Several hypotheses may explain the synergistic effects of tetracyclines and aminoglycosides. Both tetracyclines and aminoglycosides exert their antimicrobial effects by inhibiting protein synthesis. However, tetracyclines are bacteriostatic agents, which inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site (A) [24], whereas aminoglycosides are bactericidal antimicrobials, which act by impairing bacterial protein synthesis through binding to the 16S rRNA component 30S subunits of ribosomes [25]. The inhibition of protein synthesis may be enhanced by a tetracycline–aminoglycoside combination due to the disruption of multiple translation sites. There are 4 mechanisms by which bacteria can acquire resistance to tetracyclines: expression of efflux pumps, modification and degradation of drugs, ribosomal mutations, and ribosome protection proteins [24]. Among these, tetracycline-specific efflux pumps, such as Tet(A) and Tet (B), which belong to the major facilitator superfamily (MFS), and the MDR pump, AcrB, which belongs to the resistance-nodulation-division (RND) family, are the most frequent tetracycline-resistance determinants in Gram-negative bacteria [26]. However, neither tetracycline-specific efflux pumps nor AcrB can pump out aminoglycosides [25]. Therefore, cross-resistance is unlikely to occur. Moreover, aminoglycosides may disrupt and permeabilize the outer membranes of bacteria and increase the penetration of tetracyclines [27], thereby enhancing bacterial killing and suppressing the emergence of resistance.

Several limitations exist in this study. The conditions in culture medium are different than the environment in the human body. The immune system also plays an important role in defending against and resolving bacterial infections, and it may interact with antibiotics in bacterial killing. For example, previous studies have found that some cationic antimicrobial peptides enhance the activity of antimicrobial agents, and reduce the frequency of the emergence of resistant mutants [28–30]. In addition, the antibiotic concentrations in the experiments in this study remained constant, which does not simulate their pharmacokinetics in the human body. The in vitro pharmacokinetic/pharmacodynamics (PK/PD) studies which can accurately simulate the pharmacokinetics in vivo provide us more information on the effect of antibiotic combinations against MDR bacteria [31–33]. Therefore, our findings should be further evaluated with in vitro PK/PD models before application in clinical practice.

Conclusions

Due to the lack of effective antibiotics against KPC-producing *K. pneumoniae*, combination therapy seems to be a useful strategy to improve clinical effectiveness and prevent the development of resistance. We found that minocycline combined with an aminoglycoside mediated synergistic activity against KPC-producing *K. pneumoniae*, if the individual MICs were in the susceptible range. The addition of aminoglycosides at clinically relevant concentrations reduced the MPCs for minocycline, indicating that this combination effectively restricts resistance emergence. Further PK/PD and clinical studies are warranted to validate the efficacy of this drug combination.

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Compilance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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