



Original Article

In Vitro Synergistic Antimicrobial Effect of Imipenem and Colistin Against an Isolate of Multidrug-resistant *Enterobacter cloacae*

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BACKGROUND/PURPOSE: *Enterobacter cloacae* is an important nosocomial pathogen responsible for various infections. Little is known about the synergistic effects of imipenem and colistin against multidrug-resistant *E. cloacae*. Therefore, we investigated the *in vitro* effects of imipenem and colistin against a clinical isolate of multidrug-resistant *E. cloacae*.

METHODS: A strain of *E. cloacae*, designed Ent 831, was isolated from the sputum of a woman who developed severe pneumonia in a medical intensive care unit. Minimal inhibitory concentrations (MICs) of imipenem and colistin were determined by the agar dilution method. The synergistic effects were investigated using the time-kill method.

RESULTS: MICs of imipenem and colistin for *E. cloacae* strain Ent 831 were 0.5 µg/mL and 1.0 µg/mL, respectively. Using a standard inoculum (5×10^5 CFU/mL), synergism was shown with a concentration of two times the MICs of imipenem and colistin. Furthermore, four times the MIC of imipenem completely inhibited bacterial growth for more than 48 hours, but four times the MICs of colistin resulted in regrowth after 4 hours. There was no synergism between imipenem and colistin at two times the MICs against a high concentration inoculum (6.24×10^6 CFU/mL). Nevertheless, imipenem, with or without colistin, at a concentration of four times MICs could inhibit the growth of bacteria for more than 48 hours.

CONCLUSION: High-dose imipenem, alone or in combination with colistin, is effective against multidrug-resistant *E. cloacae*. Colistin alone, even at a high dose, is not effective. However, *in vitro* susceptibility to antimicrobial compounds does not always correlate with clinical success. Thus further testing of these antibiotic combinations in animal models is needed in order to predict their suitability for clinical use.

KEYWORDS: colistin, *Enterobacter cloacae*, imipenem, minimal inhibitory concentrations, time-kill study

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Introduction

Case Presentation

A 48-year-old woman with underlying acute promyelocytic leukemia was admitted to a medical intensive care unit (ICU) with acute upper gastrointestinal tract bleeding accompanied by acute respiratory failure. Suspected aspiration pneumonia developed over the left lung field after 48 hours of hospitalization. Initial sputum culture did not yield any pathogens. Empirical antimicrobial therapy with intravenous cefpirome (2 mg every 12 hours) was administered. On Day 13 of hospitalization, a new-onset ventilator-associated pneumonia occurred over the right lung field (Figure 1A). Antimicrobial therapy was changed to intravenous imipenem 500 mg every 6 hours. A sputum culture yielded *Enterobacter cloacae*, which was only susceptible to amikacin, cefpirome and imipenem, but resistant to ampicillin, cefazolin, cefuroxime, ceftazidime, ciprofloxacin, ertapenem, flomoxef, gentamicin, lomefloxacin, piperacillin and piperacillin/tazobactam, as assessed by a standard disc diffusion test.¹ The patient passed away on Day 20 of hospitalization due to worsening pneumonia (Figure 1B), even after appropriate imipenem therapy for 7 days. The sputum culture obtained on the last day of hospitalization yielded a multidrug-resistant *E. cloacae* strain with the same antibiogram as the previous isolate. The causative organism, designated strain Ent 831, was used in this study with the aim of finding potentially effective therapeutic options other than therapy with imipenem alone.

Enterobacter species are present in both adult and neonatal ICUs.²⁻⁶ Indeed, *E. cloacae* rarely cause diseases in healthy individuals. However, it is an important nosocomial

pathogen responsible for various infections, e.g. bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections, intra-abdominal infections, septic arthritis, and osteomyelitis.⁷ The resistance rate of *Enterobacter* to extended-spectrum cephalosporins was 41.2% in German hospitals.³ Specific risk factors for infection with nosocomial multidrug-resistant strains of *Enterobacter* species include the recent use of broad-spectrum cephalosporins, or aminoglycosides, and admission to the ICU.^{3,8-12} *Enterobacter* spp. that appears susceptible to cephalosporins at diagnosis may quickly develop into a resistant strain during therapy. Generally, the β -lactam rings of carbapenems and cefepime are more stable than those of third-generation cephalosporins against the AmpC β -lactamases produced by resistant strains of *Enterobacter* spp.⁷

Imipenem, a subgroup of carbapenems, has an extremely broad spectrum of activity against both Gram-positive and Gram-negative bacteria, including *E. cloacae*.¹³ As a fermentation product of the bacteria *Bacillus colistinus*, colistin was first discovered in 1949.¹⁴ The use of colistin was temporarily abandoned in the 1970s and early 1980s due to reports of a high incidence of nephrotoxicity.^{15,16} However interest in colistin was rekindled following a rise in the prevalence of multidrug-resistant Gram-negative strains and it is now widely used for the treatment and eradication of *Pseudomonas* infections in patients with cystic fibrosis.¹⁷ Colistin is bactericidal and is active *in vitro* against a broad range of Gram-negative bacteria, e.g. *Escherichia coli*, *Klebsiella* species, *Acinetobacter* species, *Enterobacter* species, and it is highly active against *Pseudomonas aeruginosa*, with reported resistance rates of less than 5%.¹⁸⁻²⁰ Based on the treatment of pneumonia

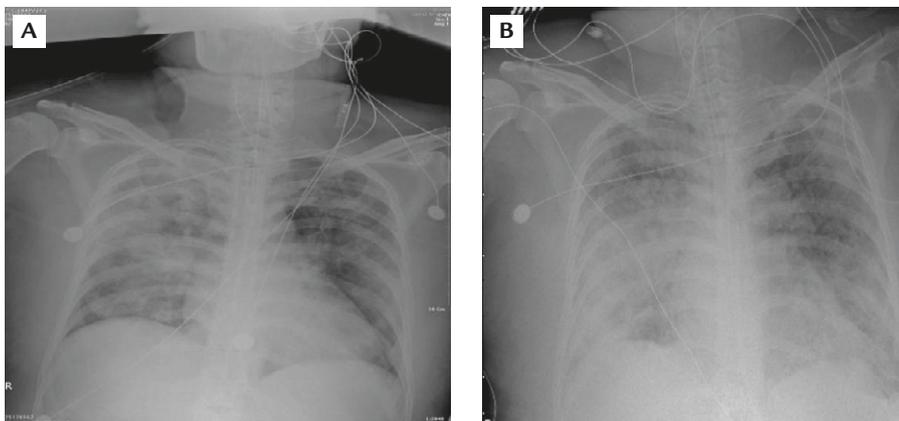


Figure 1. (A) Day 13 of hospitalization. Chest X-ray shows new patches over the right lung field in addition to residual infiltration of the left lung. The concurrent sputum culture yielded a strain of multi-drug resistant *Enterobacter cloacae*. (B) Day 20 of hospitalization. Chest X-ray shows a worsening alveolar pattern of infiltration over the right lung field. The concurrent sputum culture yielded a strain of multi-drug resistant *Enterobacter cloacae*.

caused by multidrug-resistant *E. cloacae*, both imipenem and colistin were selected to test the *in vitro* synergistic antimicrobial effects against the resistant isolate that resulted in the failure of imipenem therapy and an ultimately fatal outcome for this patient.

Methods

Bacterial isolates

E. cloacae strain Ent 831 was isolated from the sputum of a woman with acute promyelocytic leukemia in the Chi-Mei Medical Center, Tainan on June 21, 2008.

Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MICs) of cefepime (Bristol Myers Squibb Laboratories, Princeton, NJ, USA), imipenem (Merck Sharp & Dohme, West Point, PA, USA) and colistin sulfate (Sigma, St. Louis, MO, USA) were determined using agar dilution methods with Mueller-Hinton agar according to the Clinical Laboratory Standards Institute.²¹ MIC is the lowest concentration of an antibiotic resulting in the complete inhibition of visible growth of an organism. The MIC for tigecycline was performed using E-test strips (AB BIODISK, Solna, Sweden). Quality control was performed by testing *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

Determination of synergistic effects

Time-kill methods

E. cloacae Ent 831 was diluted to approximately 5×10^5 CFU/mL and 6.24×10^6 CFU/mL for standard inoculum and high inoculum experiments, respectively.²² A 100- μ L aliquot from each 10-fold serial dilution was plated on nutrient agar and bacterial colonies were counted after 0, 2, 4, 6, 8, 12, 24, 30, 36 and 48 hours. The lower limit of detection was set at 10 colonies. All experiments were performed at least twice. The inhibitory effects of imipenem and colistin, alone and in combination, against *E. cloacae* strain Ent 831 were evaluated. A bactericidal effect was defined as a $\geq 3 \log_{10}$ decrease from the starting inoculum with the effect sustained for at least 24 hours. Bacteriostatic activity was concluded if the inoculum size was maintained, or reduced by $< 3 \log_{10}$ CFU/mL, over 24 hours.

Synergism was defined as a $\geq 2 \log_{10}$ CFU/mL reduction of bacterial colonies caused by combining two compounds

when compared with the reduction numbers caused by the active single constituent after 24 hours. There was no sustained inhibitory activity against *E. cloacae* strain Ent 831 using a standard inoculum for at least 24 hours at \leq the MIC for imipenem, colistin alone, or in combination. Therefore, the drug concentrations used in the time-kill studies started at double the MIC of each antimicrobial agent. To compare the dose-dependent effect, two concentrations (2 times and 4 times) the MIC of imipenem (1.0 μ g/mL and 2.0 μ g/mL) and colistin (2.0 μ g/mL and 4.0 μ g/mL) were prepared.

Mechanism of antimicrobial resistance

Detection of the *bla*_{SHV}, *bla*_{TEM}, and *bla*_{CTX-M} genes

Polymerase chain reaction was used (with plasmid DNA as the template) to amplify the entire sequences of the *bla*_{SHV-12} (forward primer: 5'-ATG CGT TAT ATT CGC CTG TG-3' and reverse primer: 5'-TTA GCG TTG CCA GTG CTC G-3') and those of other β -lactamase genes including *bla*_{CTX-M-3}, *bla*_{CTX-M-14} and *bla*_{TEM} using specific primers as previously described.²³⁻²⁵ The purified amplicons were sequenced and analyzed online at the National Center for Biotechnology Information website.

Results

The MIC of tigecycline, cefepime, imipenem and colistin against *E. cloacae* strain Ent 831 were 4.0 μ g/mL, 2.0 μ g/mL, 0.5 μ g/mL and 1.0 μ g/mL, respectively. Polymerase chain reaction and subsequent sequence analysis confirmed the presence of *bla*_{SHV-12}.

Standard inoculum experiments

Colistin at two times the MIC completely inhibited the growth of bacteria after 4 hours of incubation. However, the bacteria regrew to the same level as the control after 24 hours (Figure 2A). Colistin at four times the MIC completely inhibited the growth of bacteria after 4 hours, but bacteria re-grew to the original inoculum size after 12 hours and reached the level of the control after 32 hours (Figure 2B).

Imipenem at two times the MIC reduced the growth of bacteria to approximately 5×10^2 CFU/mL after 8 hours of incubation. The bacteria then re-grew to reach the level of the control after 24–28 hours (Figure 2A). Imipenem at four times the MIC completely inhibited the growth of

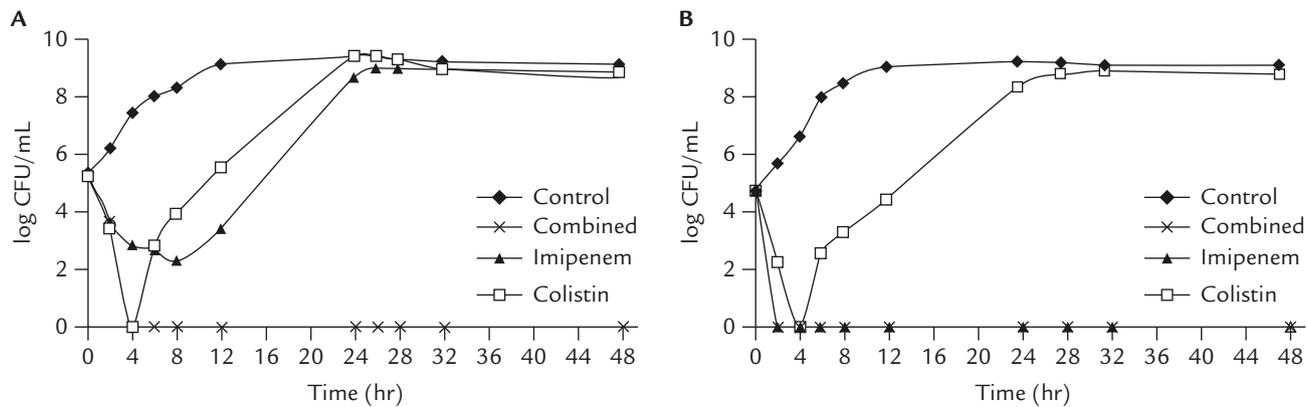


Figure 2. Time-kill study for imipenem and colistin at (A) two times and (B) four times the minimal inhibitory concentration against *Enterobacter cloacae* strain Ent 831 with a standard inoculum.

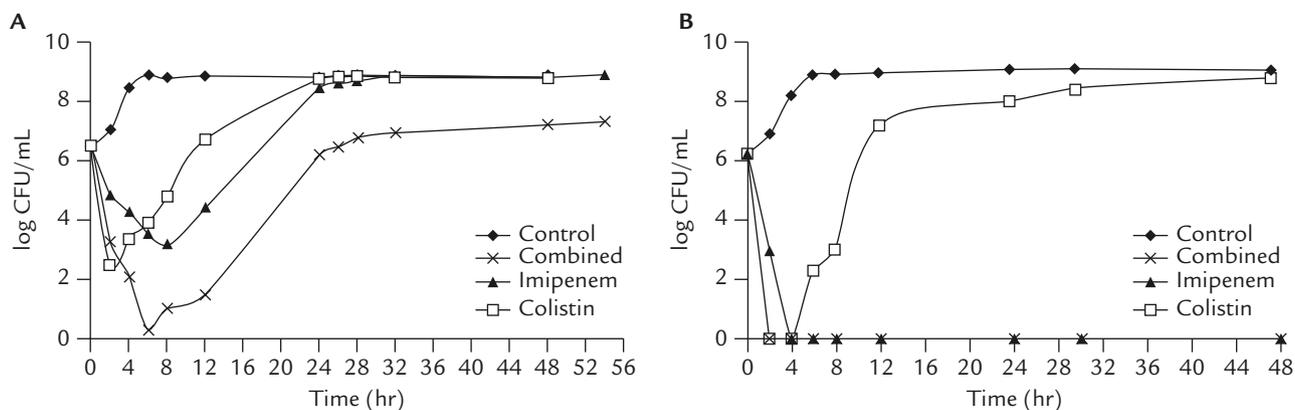


Figure 3. Time-kill study for imipenem and colistin at (A) two times and (B) four times the minimal inhibitory concentration against *Enterobacter cloacae* strain Ent 831 with a high inoculum.

bacteria after 2 hours, and this inhibitory effect persisted for more than 48 hours (Figure 2B).

A combination of imipenem and colistin at two times and four times MIC, completely inhibited bacterial growth after 4 and 2 hours, respectively. This inhibitory effect persisted for more than 48 hours.

High inoculum experiments

Colistin at two times the MIC reduced the growth of bacteria to approximately 5×10^2 CFU/mL after 2 hours. The bacteria then regrew to reach the level of the control after 24 hours (Figure 3A). Colistin at four times the MIC completely inhibited the growth of bacteria after 2 hours. The bacteria regrew to the original inoculum size after 10 hours and reached control levels after 24 hours (Figure 3B).

Imipenem at two times the MIC reduced the growth of bacteria to approximately 5×10^3 CFU/mL after 8 hours.

The bacteria regrew to reach the level of the control after 24–28 hours (Figure 3A). Imipenem at four times the MIC completely inhibited the bacterial growth after 4 hours of incubation and this inhibitory effect persisted for more than 48 hours (Figure 3B).

A combination of colistin and imipenem at two times the MIC inhibited the growth of bacteria to <50 CFU/mL after 6 hours, followed by regrowth to reach the inoculum size after 24 hours (Figure 3A). The combination of colistin and imipenem at four times the MIC completely inhibited growth after 4 hours and this inhibitory effect persisted for more than 48 hours (Figure 3B).

In summary, the use of imipenem, either with or without colistin, at four times the MIC was bactericidal against both standard and high inoculums of *E. cloacae* strain Ent 831. Colistin alone at four times the MIC was only bacteriostatic for *E. cloacae* strain Ent 831, regardless of inoculum concentration. A combination of imipenem and

colistin at two times the MIC was bactericidal for the standard *E. cloacae* strain Ent 831 inoculum. Either imipenem or colistin alone at two times the MIC were bacteriostatic for the *E. cloacae* strain Ent 831, regardless of inoculum concentration.

Discussion

Imipenem has excellent broad-spectrum activities against aerobic and anaerobic Gram-positive and Gram-negative bacteria. Colistin is regarded as being bactericidal and is active *in vitro* against a broad range of Gram-negative bacteria, e.g. *Acinetobacter* species, *Citrobacter* spp., *E. coli*, *Enterobacter* spp., *Haemophilus influenzae*, *Klebsiella* spp., *Salmonella* and *Shigella* species.¹⁹ *E. cloacae* are resistant to cephalosporins due to the mechanism of derepressive production of the ampC enzyme and/or production of extended spectrum beta-lactamase (ESBL).^{10,26-28} Resistance to carbapenems is unusual and may be due to the production of metallo- β -lactamases (MBL).²⁹ The synergistic activity of combinations of colistin and rifampin or imipenem against an MBL-producing *E. cloacae* strain has been demonstrated and this combination of three drugs successfully treated a severe *E. cloacae* infection.³⁰

The antimicrobial resistance mechanism of *E. cloacae* strain Ent 831 includes an ESBL (SHV-12) and, probably, an intrinsic AmpC β -lactamase, similar to that in previous reports.^{12,24,25} Therefore, it would be reasonable to expand these studies to cover multidrug-resistant *E. cloacae* in Taiwan. Antimicrobial susceptibility testing revealed that *E. cloacae* strain Ent 831 was susceptible to amikacin, cefpirome or cefepime, imipenem and colistin. Owing to the production of SHV-12 by *E. cloacae* strain Ent 831, the possibility of the therapeutic failure of cefpirome or cefepime against this isolate is real.^{12,24,25,31} Although tigecycline was highly active against ESBL-producing *E. cloacae* in our previous report,³² *E. cloacae* strain Ent 831 was not susceptible to tigecycline (MIC > 2 μ g/mL) in the current study, highlighting the need to continue monitoring the activity of this new compound against ESBL-producing isolates.

In this study, we used the time-kill method to evaluate the synergism antimicrobials against a multidrug-resistant strain of *E. cloacae*. The synergistic effect of imipenem and colistin was shown when both drugs were combined

at two times the MIC in standard inoculum experiments. However, a combination of both drugs at two times the MIC was only bacteriostatic in the high inoculum experiments. Regardless inoculum concentration, a high dose of imipenem (4 times MIC), either alone or in combination with colistin (4 times MIC) showed bactericidal activity. Surprisingly, colistin at both two times MIC and four times MIC was only bacteriostatic in both the standard and high inoculum experiments. Accordingly, despite its apparent susceptibility to colistin, multidrug-resistant *E. cloacae* should not be treated with colistin monotherapy.

The limited usage of colistin in the 1980s was due to reports of a high incidence of nephrotoxicity.^{15,16} Early clinical reports of severe toxicity are likely to have occurred as a result of inappropriate patient selection, higher than recommended doses and inappropriate monitoring.¹⁸ The time-kill study demonstrated the synergistic effects of antibiotics against *E. cloacae* *in vitro*. However, one limitation in this study is that we did not include an isolate that was not susceptible to imipenem or colistin. Furthermore, *in vitro* susceptibility to antimicrobial compounds does not always correlate with *in vivo* activity. In addition, the nephrotoxicity of colistin may still be a concern in clinical practice. Further testing of these antibiotic combinations in animal models is needed in order to predict their suitability for clinical use in humans.

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