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ORIGINAL ARTICLE

Enhanced efficacy of imipenem-colistin combination therapy against multiple-drug-resistant *Enterobacter cloacae*: *in vitro* activity and a *Galleria mellonella* model

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antimicrobial synergy;
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Abstract *Background/Purpose:* To investigate the *in vitro* and *in vivo* activity of imipenem-colistin combination against multidrug-resistant *Enterobacter cloacae* infections in order to determine whether it should be explored further.
Methods: The antimicrobial activity of colistin alone and in combination with imipenem was assessed versus an imipenem-susceptible isolate, *E. cloacae* GN1059, or an imipenem-resistant strain, *E. cloacae* GN0791, isolated in Anhui, China. The potential synergy of imipenem-colistin was evaluated using a checkerboard assay, as well as static time-kill experiments at 1× and 2× minimum inhibitory concentration (MIC). A simple invertebrate model (*Galleria mellonella*) was developed to assess the *in vivo* efficacy of imipenem-colistin in treating *E. cloacae* infection.
Results: In checkerboard assays, synergy (defined as a fractional inhibitory concentration index of ≤ 0.5) was observed between imipenem and colistin for both isolates tested. In time-kill assays, the combination of imipenem-colistin at 1× or 2× MIC resulted in complete killing of both strains. In the *G. mellonella* larvae model infected with lethal doses of *E. cloacae*, the

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combination therapy led to significantly increased survival of the larvae as compared with imipenem or colistin monotherapy alone ($p < 0.05$).
Conclusion: This is the first report demonstrating the efficacy of antimicrobial agents in the *G. mellonella* larvae model of infections caused by *E. cloacae*. Our study suggested that imipenem-colistin combination was highly active against *E. cloacae* both *in vitro* and in the simple invertebrate model, and provided preliminary *in vivo* evidence that such combination might be useful therapeutically.

Introduction

Enterobacter cloacae recently emerged as an important nosocomial opportunistic pathogen responsible for various nosocomial infections, including bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections, and intra-abdominal infections. *E. cloacae* has a tendency to develop resistance during the course of treatment and, hence, at least two antibiotics should be prescribed simultaneously for serious infections.[1,2]

Imipenem (IMP), a subgroup of carbapenems, has an extremely broad spectrum of activity against both Gram-positive and -negative bacteria, including *E. cloacae*. With increased reports about carbapenem-resistant *E. cloacae* clinical isolates, nosocomial life-threatening infections are only treatable with a limited number of agents, such as colistin **(COL)**.[3,4] The synergy of combinations of COL and rifampin or IMP against an metallo-β-lactamase-producing *E. cloacae* strain was reported by Tascini et al.[5] Another study showed that IMP in combination with COL was effective against multiple drug resistant (MDR) *E. cloacae*, and this combination successfully treated a case of severe *E. cloacae*-related pneumonia.[6]

Although this combination appears to be a promising treatment option based on *in vitro* data, further testing in animal models is needed in order to predict their suitability for clinical use in humans. Mammalian models are routinely employed for such studies, since the data generated are most relevant to human infections; however, their use poses significant practical, financial, and ethical barriers. As a result, invertebrate models, such as *Galleria mellonella* larvae, have been proposed as inexpensive and easy alternatives to investigate the *in vivo* activity of antimicrobial agents. A number of studies recently used this model to investigate the *in vivo* activity of antimicrobial agents versus *Acinetobacter baumannii* among other pathogens.[7–10]

Here, we employed the *G. mellonella* infection model to study the *in vivo* activity of IMP-COL combination against MDR *E. cloacae* in an attempt to determine whether it should be explored further.

Methods

Bacteria, insects, and antimicrobial susceptibility testing

Two clinical isolates of *E. cloacae* were isolated at tertiary care hospitals located in Anhui, China, in 2013. *E. cloacae* GN1059 was isolated from the blood of a patient diagnosed with brain-stem infarction and admitted to the Intensive Care Unit. *E. cloacae* GN0791 was isolated from the sputum of a patient who was admitted to the neurosurgery ward (Table 1). Batches of *G. mellonella* larvae (Kaide Ruixin Co., Ltd., Tianjin, China) in their final instar stage were stored in the dark at 4°C and used within 7 days of shipment. Larvae masses varied slightly, but were typically 250 mg, and this value was used to calculate treatment doses. All tested antibiotics, including colistin sulfate, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Minimum inhibitory concentrations (MICs) were determined by Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution methods.[11] Susceptibility was determined using CLSI breakpoints.

Synergy testing by checkerboard assay

Synergy between IMP and COL was assessed using the microtiter plate checkerboard assays as described previously.[12] In brief, 96-well microtiter plates were set up with increasing concentrations of IMP (0.06—256 mg/L) in the horizontal wells and COL (0.06—4 mg/L) in the vertical wells, and inoculated with 5×10^5 CFU/mL *E. cloacae* prepared in LB broth. Plates were incubated at 37°C for 24 hours and visually inspected for turbidity to determine growth. Synergy was assessed by the calculation of fractional inhibitory concentration index (FICI) and susceptible breakpoint index (SBPI) as previously described. SBPI was calculated as ([COL susceptibility breakpoint]/[MIC of COL in combination with IMP])+([IMP susceptibility breakpoint]/[MIC of IMP in combination with COL]). Synergy was defined as FICI ≤ 0.5 and SBPI > 2.[13]

Time-kill assays

Time-kill assays were conducted for each strain using IMP or COL alone and in combination according to a previously described methodology.[12] Each experiment was performed in duplicate to ensure reproducibility. In brief, antimicrobial regimens consisted of multiples of the MIC (1× and 2× MIC) of IMP or COL alone or in combination. LB broth was inoculated with 5×10^5 CFU/mL of the strains and incubated at 37°C. Colony counts were obtained at 0 hours, 2 hours, 4 hours, 8 hours, and 24 hours to determine the viable CFU/mL. The lower limit of detection was set at 10 colonies (1 $\log_{10}$ CFU/mL). Synergy was defined as a ≥ 2 $\log_{10}$ CFU/mL decrease between the combination and the most efficient agent alone at 24 hours. Bactericidal activity was defined as a ≥ 3 $\log_{10}$ CFU/mL reduction in cell numbers compared with the initial inoculum after 24 hours.

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Table 1 Summary of *in vitro* synergy testing and treatment efficacy in *Galleria mellonella* against two *Enterobacter cloacae* strains.

Antibiotic	MIC (mg/L)		FICI [a]		SBPI [a]		Time-kill assay results [b]		*G. mellonella* survival (%) [c]	
	GN1059	GN0791	GN1059	GN0791	GN1059	GN0791	GN1059	GN0791	GN1059	GN0791
Control	—	—	—	—	—	—	Growth	Growth	6.25 ± 6.25	4.17 ± 3.60
IMP	0.5	64	—	—	—	—	Bactericidal, regrowth at 24 h	Bactericidal, regrowth at 24 h	70.67 ± 9.60	8.33 ± 3.60
COL	0.5	1	—	—	—	—	Bactericidal, regrowth at 24 h	Bactericidal, regrowth at 24 h	56.25 ± 6.25	27.08 ± 3.60
IMP+COL	—	—	0.37	0.31	32.7	8.25	Bactericidal, no regrowth	Bactericidal, no regrowth	95.83 ± 3.60	85.42 ± 7.21

[a] As observed in three independent experiments.
[b] IMP at 2× MIC inhibited the growth of both strains, and this inhibitory effect persisted for 24 hours.
[c] *G. mellonella* survival at 96 hours after infection; values are means from three replicate experiments ± standard deviation.
COL = colistin; FICI = fractional inhibitory concentration index; IMP = imipenem; IMP+COL = imipenem-colistin combination; MIC = minimum inhibitory concentration; SBPI = susceptible breakpoint index.

G. mellonella-*killing and -treatment assays*

The *G. mellonella*-infection model was adapted from that proposed for *A. baumannii* by Peleg et al.[14] All experiments were approved in advance by the Animal Care and Use Committee of Anhui Medical University, Hefei, China. *E. cloacae* GN1059 and GN0791 were grown overnight in LB broth and washed twice in sterile phosphate buffered saline (PBS). In order to establish the inoculum required to kill *G. mellonella* over 48–96 hours, 10 larvae were inoculated with 10 μL of bacterial suspensions containing approximately 10^4 CFU/larva, 10^5 CFU/larva, and 10^6 CFU/larva of bacteria in PBS. Bacteria were injected into the hemocoels through the last left proleg using a 50-μL Hamilton syringe (Hamilton Company, Shanghai, China). Larvae were incubated in petri dishes lined with filter paper at 37°C and observed daily for 4 days. Insects were considered dead if they failed to respond to touch. For antimicrobial treatment assays, 16 larvae were infected with a lethal dose of *E. cloacae* GN1059 or GN0791 as described above. Antibiotics were administered via 10-μL injections into the last right proleg within 2 hours of inoculation. Doses were chosen to mimic those used to treat human infections and consisted of IMP at 15 mg/kg and COL at 2.5 mg/kg. IMP or COL was assessed individually, and IMP-COL combination was also assessed. Treatment was given only once. Sixteen uninjected larvae and 16 larvae injected with 10 μL of sterile PBS in place of bacteria were used as controls. To allow for the trauma associated with double injections, uninfected larvae inoculated twice with 10 μL PBS were also used. The larvae were observed for survival every 24 hours for 4 days. Experiments were performed three times on separate occasions. The survival rate (and standard deviation) of larvae at the 96-hour assay endpoint was calculated using pooled data across replicate experiments.

Statistical analysis

All statistical analyses were performed using GraphPad Prism, version 5.04 (GraphPad Software Inc., San Diego, CA, USA). Survival curves were analyzed using the log-rank test, with a $p \leq 0.05$ indicating statistical significance.

Results

Antimicrobial susceptibility

E. cloacae GN1059 was susceptible to all agents, including β-lactams, aminoglycosides, quinolones, COL, and tigecycline. The MIC of COL against GN1059 was 0.5 mg/L. GN0791 was resistant to all agents tested, but susceptible to COL (MIC = 1 mg/L) and tigecycline. The MIC of IMP against GN1059 and GN0791 was 0.5 mg/L and 64 mg/L, respectively.

Checkerboard assays

In microtiter checkerboard assays, the presence of COL at 0.125 mg/L reduced the IMP MIC from 0.5 mg/L to 0.06 mg/L against GN1059. For GN0791, the presence of COL at 0.25 mg/L reduced the IMP MIC from 64 mg/L to 4 mg/L. A FICI < 0.5 was observed for both strains, indicative of a potent synergistic interaction. An SBPI > 2 was also repeatedly seen for both strains, providing further evidence of the strength and clinical relevance of the combination (Table 1).

Time-kill assays

COL at 1× or 2× MIC completely inhibited the growth of GN1059 or GN0791 after 4 hours incubation, but did not provide sustained killing over 24 hours, despite the apparent susceptibility of both strains to COL in static assays (Figure 1). IMP at 1× MIC reduced the growth of GN1059 or GN0791 to ~2 $\log_{10}$ CFU/mL after 8 hours incubation. However, both strains then regrew to reach the level of the control after 24 hours (Figures 1A and 1C). IMP at 2× MIC completely inhibited the growth of both strains after 2 hours, and this inhibitory effect persisted for 24 hours (Figures 1B and 1D).

By contrast, the combination of IMP and COL at 1× or 2× MIC displayed both rapid and consistently bactericidal activity, resulting in complete killing without regrowth over the time course of the assay for each of the strains.

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E. cloacae—G. mellonella *kill kinetics*

Both GN1059 and GN0791 were pathogenic to *G. mellonella* at $> 10^4$ CFU/larva. Most of the killing occurred in the first 24 hours, followed by further killing in the subsequent 3 days. The mortalities at 4 days were 100% at 10^6 CFU/larva and between 70% and 90% at 10^5 CFU/larva. Based on these data, 5×10^5 CFU/larva of both strains was selected as the inoculum for subsequent treatment experiments.

Activities of IMP and COL in the *G. mellonella* model

Administration of COL protected *G. mellonella* from GN1059-mediated killing, as > 56% of the larvae were still alive at 96 hours postinoculation. However, COL monotherapy performed poorly as compared with the IMP-COL combination versus GN0791 (Figure 2B). The survival rate of COL to GN0791-infected larvae was 27.08% (± 3.60) as compared with 56.25% (± 6.25) when COL was used to treat GN1059-infected caterpillars ($p < 0.05$; Table 1). IMP was effective versus the IMP-susceptible isolate GN1059; however, when IMP was used to treat GN0791 infections, survival rates were equivalent to those for the untreated controls as predicted from the *in vitro* susceptibility data (Table 1, Figure 2A).

The combination of COL with IMP was highly effective in protecting larvae from GN1059 or GN0791 lethal infections, with survival > 85% in both cases, and was obviously superior to COL monotherapy in the treatment of GN0791 ($p < 0.05$; Table 1, Figure 2).

Discussion

Carbapenem-resistant Enterobacteriaceae (CRE) continue to emerge as a serious public health threat worldwide.[15–17] With the increased resistance to carbapenem, unorthodox combination therapies are increasingly being considered. Recently, Lin et al[6] reported that high-dose IMP alone or in combination with COL was effective against a multidrug-resistant, IMP-susceptible *E. cloacae*. Additionally, the *in vitro* synergistic effect of this combination was also shown; however, one limitation of their study was that they did not include an isolate that was not susceptible to IMP.[6]

In our study, we used the checkerboard assay and time-kill method to evaluate synergistic effects against IMP-susceptible and -resistant strains of *E. cloacae*. In checkerboard assays, synergy between COL and IMP was repeatedly observed, with FICIs < 0.5 and SBPIs > 2. A reduction of IMP MIC in the presence of sub-inhibitory concentrations of COL was observed against both strains. SBPI is a novel parameter that relates the magnitude of the interaction to the pharmacodynamic breakpoints used to determine susceptibility *in vivo*. An SBPI > 2 indicates that the agents are more active in combination than when used alone.[13] Surprisingly, COL at 1× or 2× MIC was initially bactericidal, but significant regrowth was observed at 24 hours, despite the apparent susceptibility of both strains to COL *in vitro*. This

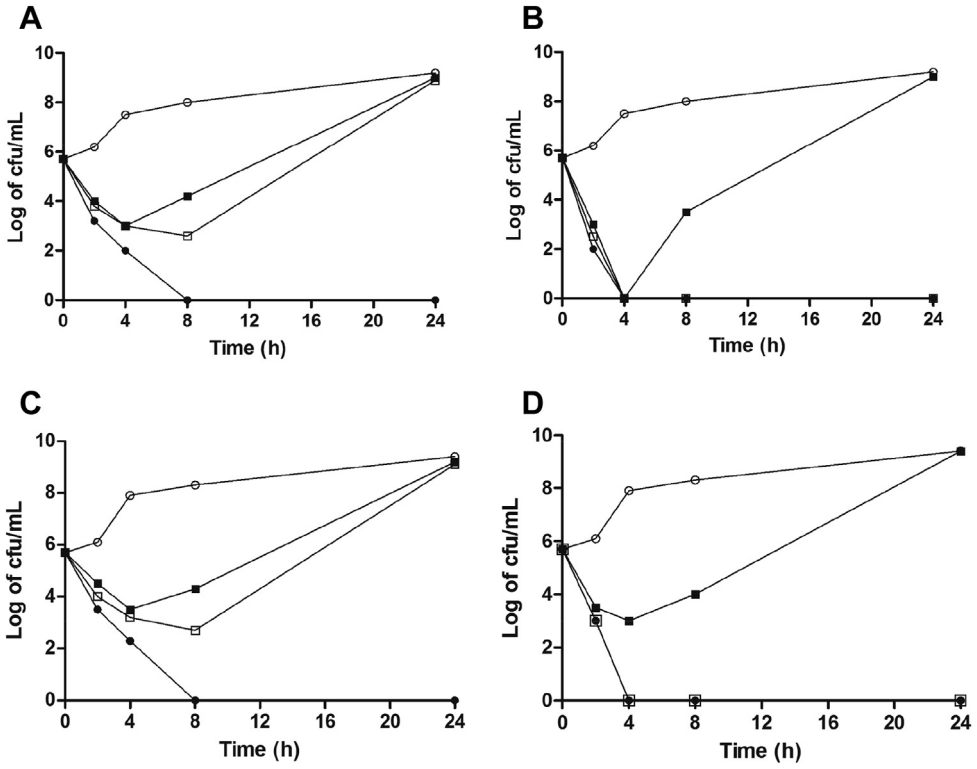

Figure 1. Time-kill assay performed on (A,B) *Enterobacter cloacae* GN1059 or (C,D) GN0791 using imipenem or colistin at (A,C) 1× MIC or (B,D) 2× MIC and an imipenem-colistin combination. Key: open circles, control; filled circles, imipenem-colistin combination; open squares, imipenem; filled squares, colistin. Data from a single representative experiment. MIC = minimum inhibitory concentration.

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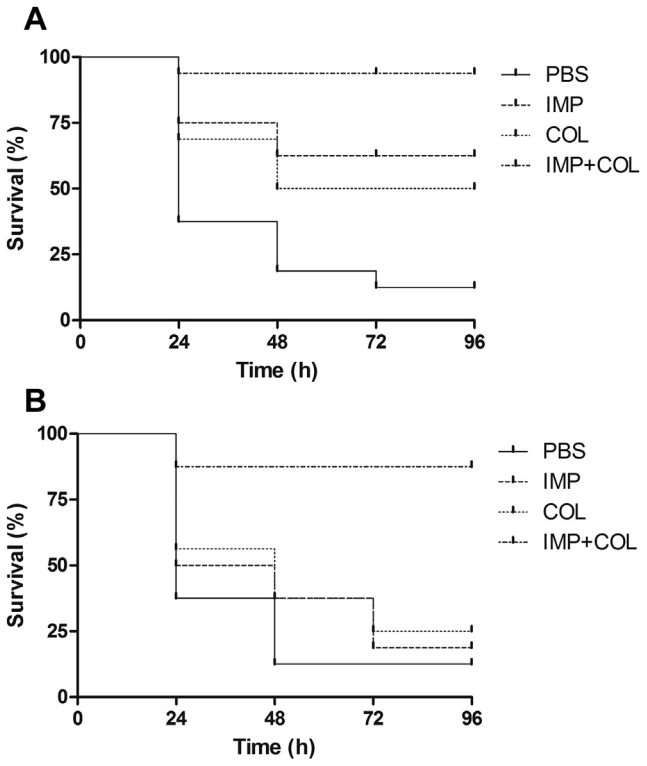

Figure 2. Survival curves for *Galleria mellonella* larvae inoculated with a lethal dose of (A) *Enterobacter cloacae* GN1059 or (B) GN0791 following treatment with imipenem (15 mg/kg), colistin (2.5 mg/kg), or an imipenem-colistin combination. Curves represent a single experiment performed using 16 insects. COL = colistin; IMP = imipenem; IMP+COL = imipenem-colistin combination; PBS = phosphate-buffered saline.

phenomenon can be explained by "colistin heteroresistance" as described previously.[18,19] Although a formal population analysis was not performed in our study, it seems likely that COL exposure selected for a heteroresistant subpopulation during the course of the time-kill study. Accordingly, despite its apparent susceptibility to COL, MDR *E. cloacae* should not be treated with COL monotherapy. IMP at 1× MIC also did not provide sustained killing over 24 hours. The reason for this may be similar to "colistin heteroresistance", but be called "imipenem heteroresistance". Some studies reported that imipenem heteroresistance can be induced by IMP in *A. baumannii* and *Klebsiella pneumoniae* strains.[20–22] By contrast, the IMP-COL combination displayed both rapid and sustained bactericidal activity at 1× or 2× MIC over the time course of the time-kill assay for each strain. The synergistic effect of the IMP-COL combination was reported in many studies, especially against *A. Baumannii*.[6,23] To our knowledge, there is only one study reporting the *in vitro* synergy of IMP-COL combination against a IMP-susceptible *E. cloacae*,[6] and our results were in line with their findings; however, the synergy effect was also found against a IMP-resistant *E. cloacae* in our study.

G. mellonella larvae have recently been used as an alternative to vertebrates as an invertebrate model host for studying a number of important human pathogens, including Gram-positive and -negative bacteria and several pathogenic fungi.[7–10] These insects have sophisticated cellular and humeral defenses, which are similar to the innate immune response of mammals, making them highly attractive for the study of acute bacterial infections.[24] We employed this model to study the *in vivo* activity of IMP-COL combination against *E. cloacae* for the first time in order to predict their suitability for clinical use in humans. The combination of IMP and COL was significantly more effective than either drug alone versus IMP-resistant *E. cloacae* when assessed *in vivo* using the *G. mellonella* larvae model of infection. This was in agreement with our *in vitro* study and provided preliminary *in vivo* evidence that such a combination might be useful therapeutically.

Although our data suggested and somewhat confirmed that IMP-COL combination demonstrated synergy effects against IMP-susceptible or -resistant *E. cloacae* infections, these results should be considered with care for at least two reasons. First, the *in vitro* and *in vivo* synergy of antibiotic combinations is often strain- and clone-specific, and we just investigated two clinical isolates. Finally, although preliminary evidence of *in vivo* efficacy can be obtained through the use of invertebrate-infection models, additional studies are required using mammalian-infection models, since it is considered that the data generated are most relevant to human infections.

In summary, our *in vitro* and *in vivo* findings reported here suggested that IMP-COL combination may be an effective therapeutic option for the treatment of *E. cloacae* infections, although additional studies are required.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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