

Antibacterial Activity of Eravacycline Against Carbapenem-Resistant Gram-Negative Isolates in China: An in vitro Study

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Objective: Eravacycline is a novel, fully synthetic fluorocycline antibiotic being developed for the treatment of serious infections, with a broad-spectrum antimicrobial activity, including against carbapenem-resistant gram-negative bacteria (CRGNB). However, the in vitro activity of eravacycline against CRGNB has not been well known in China. In this study, we analysed the antibacterial activity of eravacycline against CRGNB isolates in order to provide a theoretical basis for the clinical treatment.

Methods: A total of 346 isolates of CRGNB were collected from two different tertiary care hospitals in Zhejiang, China. Carbapenem resistance genes of all isolates were detected by polymerase chain reaction. And we analysed the in vitro activity of eravacycline against CRGNB by antimicrobial susceptibility tests. In addition, the time-kill curves were generated to evaluate the antibacterial effect of tigecycline and eravacycline.

Results: Four different types of carbapenem-resistant isolates were collected, including 50 *Escherichia coli* isolates, 160 *Klebsiella pneumoniae* isolates, 42 *Enterobacter cloacae* complex isolates, and 94 *Acinetobacter baumannii* isolates. The carbapenem resistance genes were identified in 346 isolates, including *bla*_{KPC-2} (48.0%), *bla*_{OXA-23} (27.2%), *bla*_{NDM-1} (23.1%), and *bla*_{NDM-16} (0.3%). The antimicrobial susceptibility testing results showed that the minimum inhibitory concentration (MIC) values of 346 isolates were within the sensitivity range ($\leq 0.0625\sim 16$ mg/L) and that the MIC₅₀ or MIC₉₀ of eravacycline was generally approximately 2-fold lower than tigecycline. In addition, the time-kill curves showed that the bactericidal effect of eravacycline was stronger than that of tigecycline against four different types of isolates.

Conclusion: Our research indicated that eravacycline had a good antibacterial effect on CRGNB, which could provide a theoretical basis for the clinical treatment of drug-resistant bacterial infections in the future.

Keywords: eravacycline, tigecycline, carbapenem-resistant, gram-negative isolates, drug sensitivity, time-kill curves

Introduction

Carbapenem-resistant gram-negative bacteria (CRGNB) strains are highly prevalent around the world and were ranked as the highest priority threat by the World Health Organization (WHO) in 2017.¹ In China, the incidence of carbapenem-resistant *Enterobacteriaceae* infections alone is 4.0/10,000, much higher than in other countries.^{2,3} Despite major preventative efforts, CRGNB continue to spread rapidly worldwide and pose a considerable threat to public health.⁴ A systematic meta-analysis reported that the mortality rate attributed to carbapenem-resistant *Enterobacteriaceae* infection is up to 50%,⁵ especially

bloodstream infections.⁶ Furthermore, the morbidity and mortality of patients infected with carbapenem-resistant pathogens are significantly higher than those infected with antibiotic-sensitive pathogens in the clinic, which may be due to the poor antibacterial effect of the antibiotics.^{7,8} Tigecycline, colistin, and ceftazidime-avibactam have become the last choice for the treatment of infections caused by CRGNB.⁹ With the application of these drugs, the identification of resistant isolates has begun to increase. Notably, some isolates even have multidrug-resistant phenotypes. For example, strains coharboring tigecycline-, colistin-, and ceftazidime-avibactam-resistance phenotypes have been identified,¹⁰ which will undoubtedly accelerate the formation of “superdrug bacteria”. Thus, for infections caused by multidrug-resistant bacteria, the options for antimicrobial therapy are very limited. There is an urgent need to develop new drugs or combinations thereof for the treatment of infection by these “superbugs”.

Eravacycline, a novel fully synthetic fluorocycline antibiotic, has broad-spectrum antimicrobial activity against antibiotic-resistant pathogens, including gram-positive cocci, gram-negative bacilli (except *Pseudomonas aeruginosa* and *Burkholderia* spp.), anaerobes, atypical bacterial pathogens and *Neisseria gonorrhoeae*.^{11,12} This drug was approved in 2014 by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to treat complicated intra-abdominal infections, complicated urinary tract infections (cUTIs), and pulmonary infections.^{11,13} Similar to tetracyclines, eravacycline can bind to the 30S subunit of the bacterial ribosome and inhibit bacterial protein synthesis,¹⁴ but the antibacterial effect can be enhanced by two key modifications in the chemical structure, the fluorine atom in the C-7 side chain and the pyrrolidine acetamide group in C-9.¹⁵ The chemical structures of eravacycline and tigecycline were shown in Figure 1. Therefore, it may have advantages in terms of in vitro activity, pharmacokinetics, and tolerability.¹⁶ An impressive safety profile has been demonstrated among members of this class in Phase I to III trials.¹⁴ However, eravacycline susceptibility in a large number of CRGNB in China is largely unknown.

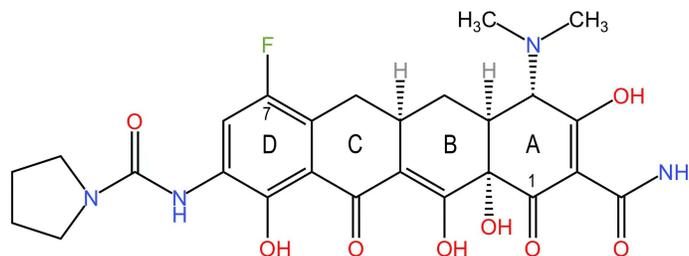
The objective of the current study was to evaluate the in vitro activities of eravacycline against CRGNB. In addition, we compared the bactericidal effect of eravacycline and tigecycline on CRGNB by time-kill curves.

Materials and Methods

Bacterial Isolates

A total of 346 CRGNB isolates were collected from two tertiary hospitals in Hangzhou, including 50 *E. coli* isolates, 160 *K. pneumoniae* isolates, 42 *Enterobacter cloacae* complex isolates, and 94 *A. baumannii* isolates. All isolates were identified using a VITEK MS mass spectrometer (bioMérieux, Marcy l’Etoile, France).

Eravacycline



Tigecycline

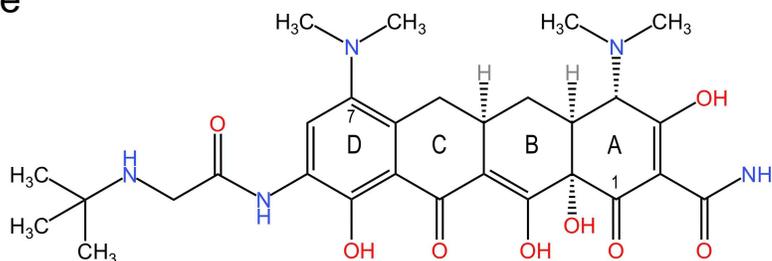


Figure 1 Chemical structures of eravacycline and tigecycline.

Detection of Carbapenem Resistance Genes

All isolates were tested for the presence of carbapenem resistance genes by polymerase chain reaction (Biometra GmbH, Germany) with specific primers and conditions as previously described.^{17,18} The amplification parameters were, initial denaturation at 95 °C for 10 min, 36 cycles of 95 °C for 30s, 52 °C for 30s and 72 °C for 50s, with 6 min at 72 °C for final extension. The amplification products were analysed by electrophoresis on 1.0% agarose gels.

Antimicrobial Susceptibility

Antimicrobial susceptibility testing was performed by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.¹⁹ All isolates tested were stored in a –80 °C refrigerator. Eravacycline and tigecycline (MedChemExpress, Shanghai, China) were freshly prepared in sterile water on the day of use. The final inoculum of 1.5×10^8 CFU/mL was acquired by adjusting the turbidity of the incubated mixture to 0.5 McFarland standards using normal saline.²⁰ Aliquots (100 µL) of the colony suspension were inoculated into 96-well broth microdilution panels (Corning, NY, USA) containing 200 µL aliquots of eravacycline and tigecycline (<0.0625, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 mg/L). And 96-well broth microdilution panels were read visually after incubation for 16–20 hours at 37 °C. The results of this study were interpreted according to the criteria of the European Committee on Antimicrobial Susceptibility (EUCAST) (<http://www.eucast.org>) and the Food and Drug Administration (FDA) (<https://www.fda.gov/>).^{21,22} *E. coli* ATCC 25922 was used as a quality control strain.

Time-Kill Curves

A time-kill assay was performed in triplicate on four representative isolates as described in a previous study.²³ Based on all minimum inhibitory concentration (MIC) values of the four types of strains, a strain with the same MIC for tigecycline and eravacycline was selected. The MIC of the isolate was used as the reference concentration of the antibiotic.^{24–26} Briefly, isolates were incubated in Mueller-Hinton broth (Thermo Fisher, Shanghai, China) to mid-log phase (OD₆₀₀ of 0.25 to 0.3), at which point antibiotics at concentrations of 1 ×, 2 ×, and 4 × MIC were added (*P*-values <0.05, Two-way Repeated Measures ANOVA), followed by shaking at 37 °C. Subsequently, culture samples were removed at 0, 2, 4, 6, 8, and 24 hours, serially diluted with saline, plated on Mueller-Hinton agar (Bio-Kont, Wenzhou, China), and incubated for 16–18 hours at 37 °C to determine the number of CFU/mL and to plot the time-kill curves.

Statistical Analysis

The extracted data were entered into Excel version 19 and exported to SPSS version 26 for data analysis. The cumulative percentage of MIC and antimicrobial susceptibility were calculated for 346 isolates using SPSS version 26. The Wilcoxon Signed Ranks Test was used to analyse the significance of the differences between groups. The Two-way Repeated Measures ANOVA was used to analyse the data of the time-kill curves. *P*-values <0.05 was considered to be significant. Figures were drawn using GraphPad Prism 8 and KingDraw.

Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki and was reviewed and approved by the Research Ethics Committee of Zhejiang Provincial People's Hospital (QT2022307).

Results

Carbapenem Resistance Genes

The carbapenem resistance genes of the 346 carbapenem isolates are shown in Table 1. Of the 50 *E. coli* isolates, 47 isolates were *bla*_{NDM-1}, one isolate was *bla*_{NDM-16}, and 2 isolates were *bla*_{KPC-2}. Of the 160 *K. pneumoniae* isolates all carried *bla*_{KPC-2}. Of the 42 *E. cloacae* complex isolates, 33 isolates were *bla*_{NDM-1}, 4 isolates were *bla*_{KPC-2}, and 5 isolates carried other carbapenem resistance genes. And 94 *A. baumannii* isolates were all *bla*_{OXA-23}.

Table 1 Carbapenem Resistance Genes of 346 Carbapenem-Resistant Isolates

Isolates	Carbapenem Resistance Genes	No. of Isolates
<i>E. coli</i> (n=50)	<i>bla</i> _{NDM-1}	47
	<i>bla</i> _{NDM-16}	1
	<i>bla</i> _{KPC-2}	2
<i>K. pneumoniae</i> (n=160)	<i>bla</i> _{KPC-2}	160
<i>E. cloacae</i> complex (n=42)	<i>bla</i> _{NDM-1}	33
	<i>bla</i> _{KPC-2}	4
	Other	5
<i>A. baumannii</i> (n=94)	<i>bla</i> _{OXA-23}	94

Abbreviations: NDM, New Delhi Metallo- β -lactamase; KPC, *K. pneumoniae* Carbapenemase; OXA, Oxacillinases.

MIC Distribution

The cumulative percentage and the MIC distributions of 346 selected carbapenem-resistant bacteria tested with eravacycline and tigecycline are shown in Tables 2–3.

For carbapenem-resistant *E. coli* (CRE) isolate, the MIC₅₀ and MIC₉₀ for both eravacycline and tigecycline were 0.25 mg/L and 0.5 mg/L, respectively. The MIC distribution range of eravacycline was ≤ 0.0625 to 4 mg/L and that of tigecycline was ≤ 0.0625 to 1 mg/L. The results showed that tigecycline had a lower MIC distribution than eravacycline for CRE isolates.

For CRKP isolates, the MIC₅₀ and MIC₉₀ for eravacycline were 0.5 mg/L and 2 mg/L, respectively. The MIC₅₀ and MIC₉₀ for tigecycline against CRKP isolates were 1 mg/L and 2 mg/L, respectively. Eravacycline and tigecycline had the same MIC distribution against *K. pneumoniae* and *A. baumannii* (≤ 0.0625 to 16 mg/L).

For carbapenem-resistant *E. cloacae* complex (CREC) isolates, the MIC₅₀ and MIC₉₀ for eravacycline were 1 mg/L and 4 mg/L, respectively. The MIC₅₀ and MIC₉₀ of tigecycline against these CREC isolates were 0.5 mg/L and 4 mg/L,

Table 2 Cumulative Percentage of MIC Values of Eravacycline and Tigecycline (%)

Antibiotics	MIC (mg/L)	<i>E. coli</i> (n=50)	<i>K. pneumoniae</i> (n=160)	<i>E. cloacae</i> Complex (n=42)	<i>A. baumannii</i> (n=94)
Eravacycline	≤ 0.0625	4.0	1.9	0	5.3
	0.125	20.0	6.9	0	7.4
	0.25	50.0	23.8	14.3	10.6
	0.5	92.0	53.1	45.2	29.8
	1	98.0	81.3	66.7	63.8
	2	98.0	96.3	81	84
	4	100	96.9	95.2	94.7
	8	100	99.4	100	98.9
	16	100	100	100	100
Tigecycline	≤ 0.0625	22.0	2.5	0	4.3
	0.125	46.0	3.1	0	7.4
	0.25	74.0	10.0	21.4	9.6
	0.5	94.0	30.6	52.4	17.0
	1	100	58.1	76.2	37.2
	2	100	92.5	85.7	72.3
	4	100	96.9	95.2	85.1
	8	100	97.5	100	98.9
	16	100	100	100	100

Abbreviation: MIC, Minimum Inhibitory Concentration.

Table 3 Drug Sensitivity Distribution of 346 Carbapenem-Resistant Isolates to Eravacycline and Tigecycline (Mg/L)

Isolates	No. of Isolates	Eravacycline			Tigecycline		
		MIC ₅₀	MIC ₉₀	MIC Range	MIC ₅₀	MIC ₉₀	MIC Range
<i>E. coli</i>	50	0.25	0.5	≤0.0625~4	0.25	0.5	≤0.0625~1
<i>K. pneumoniae</i>	160	0.5	2	≤0.0625~16	1	2	≤0.0625~16
<i>E. cloacae</i> complex	42	1	4	0.25~8	0.5	4	0.25~8
<i>A. baumannii</i>	94	1	4	≤0.0625~16	2	8	≤0.0625~16

Abbreviations: MIC, Minimum Inhibitory Concentration; MIC₅₀, Minimum inhibitory concentrations for 50% of the organisms; MIC₉₀, Minimum inhibitory concentrations for 90% MIC of the organisms.

respectively. In addition, the same MIC distribution (0.25 mg/L to 8 mg/L) for tigecycline and eravacycline was observed in the CREC isolates.

For carbapenem-resistant *A. baumannii* (CRAB) isolates, the MIC₅₀ and MIC₉₀ for eravacycline were 1 mg/L and 4 mg/L, and the MIC₅₀ and MIC₉₀ for tigecycline were 2 mg/L and 8 mg/L. The results showed that *A. baumannii* had lower MIC₅₀ and MIC₉₀ values for eravacycline than for tigecycline.

Antimicrobial Susceptibility

Table 4 provides details on the in vitro activities of eravacycline and tigecycline agents against CRE, CRKP, CREC, and CRAB isolates, including percent susceptibility according to the EUCAST and FDA breakpoints. A total of 6.0% of *E. coli*, 3.1% of *K. pneumoniae*, 4.8% of *E. cloacae* complex, and 14.9% of *A. baumannii* isolates were tigecycline-resistant. Tigecycline had a high antibacterial activity against *Enterobacteriaceae*, and the susceptibility rate was greater than 90%. The susceptibility of *E. coli* was 92% to eravacycline and 94% to tigecycline. The susceptibility of *K. pneumoniae* and the *E. cloacae* complex to eravacycline was 53.1% and 45.2%, respectively. Since *A. baumannii* did not have breakpoints for eravacycline, the sensitivity rate could not be calculated.

Time-Kill Curves Assay

Four selected CRGNB in vitro were evaluated by time-kill curves to compare the bactericidal effect between eravacycline and tigecycline (Figure 2).

Isolate CRE4032 had the same MIC for eravacycline and tigecycline (0.25 mg/L), and the time-kill curves showed that 1 ×, 2 ×, and 4 × MIC of tigecycline and eravacycline showed stable bactericidal or inhibitory effects against it (Figure 2A and B).

Table 4 Sensitivity Analysis of 346 Carbapenem-Resistant Isolates to Eravacycline and Tigecycline

Isolates	Antibiotics	% Susceptible				MIC(mg/L)		
		S	I	R	S+I	S	I	R
<i>E. coli</i> ^{a,b} (n=50)	Tigecycline	94.0 (47/50)	–	6.0 (3/50)	94.0 (47/50)	≤0.5	–	>0.5
	Eravacycline	92.0 (46/50)	–	8.0 (4/50)	92.0 (46/50)	≤0.5	–	>0.5
<i>K. pneumoniae</i> ^b (n=160)	Tigecycline	92.5 (148/160)	4.4 (7/160)	3.1 (5/160)	96.9 (155/160)	≤2	4	≥8
	Eravacycline	53.1 (85/160)	–	–	53.1 (85/160)	≤0.5	–	–
<i>E. cloacae</i> complex ^b (n=42)	Tigecycline	85.7 (36/42)	9.5 (4/42)	4.8 (2/42)	95.2 (40/42)	≤2	4	≥8
	Eravacycline	45.2(19/42)	–	–	45.2(19/42)	≤0.5	–	–
<i>A. baumannii</i> ^b (n=94)	Tigecycline	72.3 (68/94)	–	14.9 (14/94)	72.3 (68/94)	≤2	–	≥8
	Eravacycline	–	–	–	–	–	–	–

Notes: ^aThe breakpoint of *E. coli* is based on that suggested by the EUCAST. ^bThe breakpoints of *K. pneumoniae*, *E. cloacae*, and *A. baumannii* are based on those suggested by the FDA. The susceptibility breakpoint of eravacycline against *E. coli* is ≤0.5 mg/L, based on that suggested by the FDA. The breakpoints of tigecycline against *E. coli* were S: ≤2 mg/L, I: 4 mg/L, and R: ≥8 mg/L, based on those suggested by the FDA.

Abbreviations: –, not defined; S, Susceptible; I, Intermediate; R, Resistant; MIC, Minimum Inhibitory Concentration.

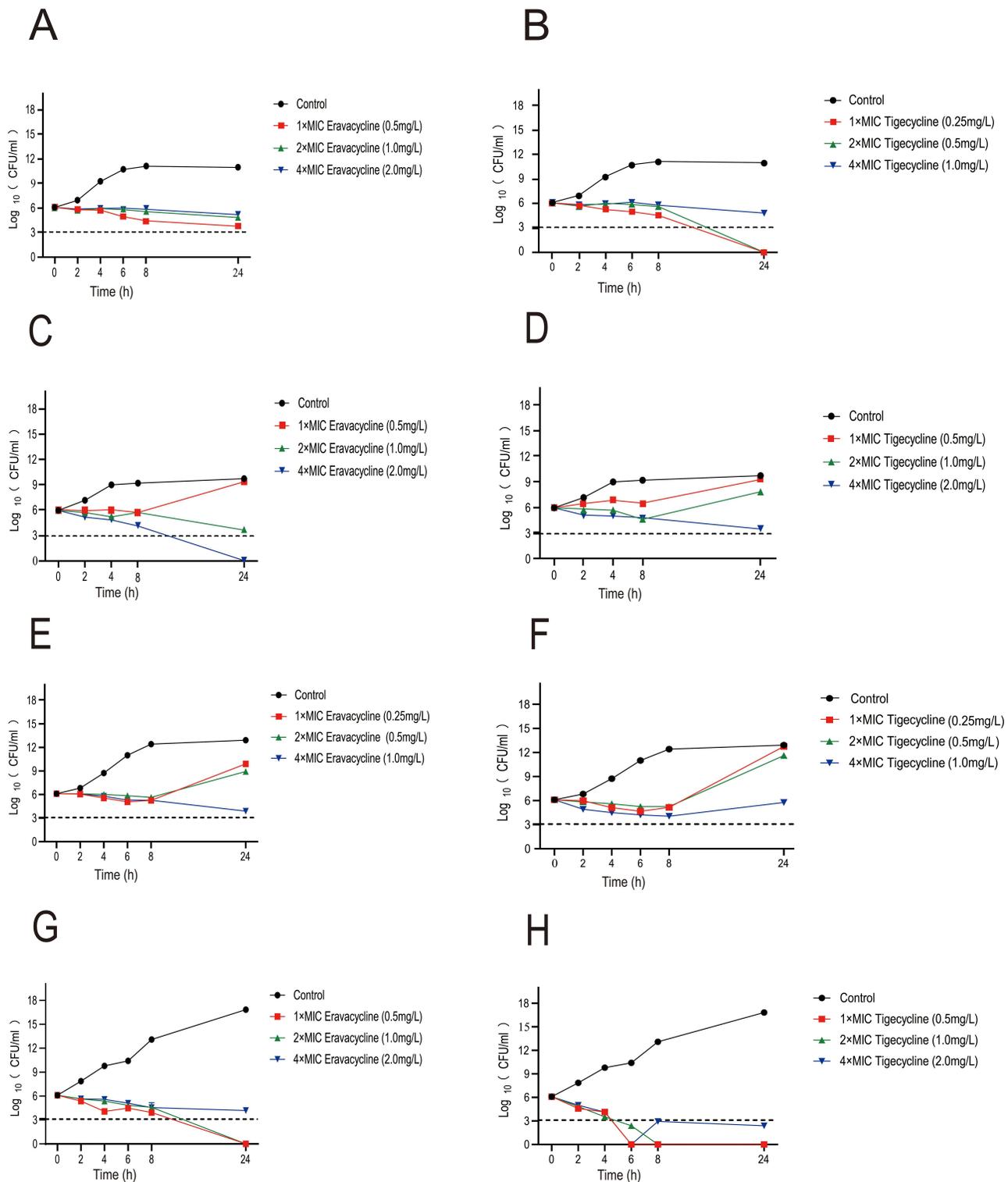


Figure 2 Time-kill curves of four types of carbapenem-resistant bacteria against eravacycline and tigecycline. (**A** and **B**): Isolate CRE4032. (**C** and **D**): Isolate CRKP5146. (**E** and **F**): Isolate CREC5512. (**G** and **H**): Isolate CRAB4124. The dashed line indicates the lower limit of detection.

Abbreviations: CRE, carbapenem-resistant *E. coli*; CRKP, carbapenem-resistant *K. pneumoniae*; CREC, carbapenem-resistant *E. cloacae* complex; CRAB, carbapenem-resistant *A. baumannii*; CFU, colony-forming unit.

Isolate CRKP5146 had the same MIC for eravacycline and tigecycline (0.5 mg/L). The time-kill curves showed that it was inhibited by 1 × and 2 × MIC of tigecycline for the first 8 hours compared to the initial counts and then resumed a normal growth rate and exceeded the initial inoculum, while 4 × MIC of tigecycline stably inhibited its growth rate. For

eravacycline, 1 × MIC inhibited this isolate, although it resumed a normal growth rate after 8 hours, while 2 × and 4 × MIC of eravacycline had a stronger bactericidal effect (Figure 2C and D). The results indicated that eravacycline exhibited a better bacterial inhibitory effect against CRKP than tigecycline.

Isolate CREC5512 had the same MIC for eravacycline and tigecycline (0.25 mg/L), and the time-kill curves showed that 1 ×, 2 ×, and 4 × MIC of tigecycline had a good inhibitory effect in the first 8 hours, after which normal growth resumed in all cases. The 1 ×, 2 ×, and 4 × MIC of eravacycline inhibited this isolate; at 1 × and 2 × MIC, this isolate resumed growth after 8 hours at a slow rate, while the 4 × MIC maintained a stable inhibitory effect (Figure 2E and F). The results indicated that eravacycline had a better inhibitory effect than tigecycline.

Isolate CRAB4124 had the same MIC for eravacycline and tigecycline (0.5 mg/L), and the time-kill curves showed that at 1 × and 2 × MIC of tigecycline, the bacterial solution concentration decreased to 0 after 6 and 8 hours, respectively. At 4 × MIC of tigecycline, the bacterial solution concentration decreased to 0 after six hours, followed by regrowth, but tigecycline still exerted a strong bactericidal effect. Eravacycline at 1 ×, 2 ×, and 4 MIC had a robust bactericidal effect on this isolate (Figure 2G and H). The results indicated that both eravacycline and tigecycline had a good bactericidal effect on CRAB.

In the time-kill curves, the results showed that eravacycline and tigecycline did not achieve complete bactericidal effects in long-term treatment. Some of these strains appeared to show evidence of transient bactericidal activity after treatment with high concentrations of eravacycline or tigecycline, but they returned to normal or slow growth after 8 hours. However, all isolates treated with eravacycline and tigecycline achieved early bactericidal effects, even at lower concentrations.

Discussion

In the present study, we investigated 346 strains containing four different types of carbapenem-resistant strains, which fell within the range (≤ 0.0625 to 16 mg/L) indicating drug sensitivity. Previous study results indicated that most carbapenem-resistant *Enterobacteriaceae* and CRAB isolates had an MIC range of ≤ 0.06 to 8 mg/L for eravacycline.^{27,28} Our results are similar to those reported above.

The FDA susceptibility breakpoint is ≤ 0.5 mg/L for eravacycline against *E. coli*.²² Our data suggested that the MIC₉₀ of eravacycline against *E. coli* was 0.5 mg/L. The results indicated that eravacycline had robust in vitro antibacterial activity. In addition, a recent study suggested that *K. pneumoniae* isolates from China have a higher MIC for eravacycline than isolates from Europe, USA, and Canada.^{12,29} In this study, the MIC₉₀ for eravacycline was 2.0 mg/L against *K. pneumoniae*, which is consistent with this statement. This may be due to the expression of efflux pumps, resistance mutations, and environmental and regional differences.^{12,29–32} In a previously published study, we reported two plasmids co-harboring the *tmexCD2-toprJ2* gene cluster and carbapenem resistance genes in two clinical ST11 CRKP strains. In addition, we identified tigecycline resistance gene clusters in CRKP strains carrying *bla*_{NDM-1} or *bla*_{KPC-2}. The spread of these resistance gene clusters in CRKP poses a substantial threat in the clinical therapeutic setting and exacerbates the antimicrobial resistance crisis.¹⁰ Notably, Table 3 shows that the MIC₅₀ and MIC₉₀ of eravacycline were 2-fold lower than those of tigecycline, especially CRAB, in accordance with previous studies.^{12,15,33,34} In short, the in vitro activity of eravacycline is much more potent than that of tigecycline. This phenomenon is attributed to the modification of two key positions in the structure of eravacycline, resulting in enhanced antimicrobial potency in vitro.^{15,35}

In the time-kill curves of tigecycline, we also found that CRKP5146 and CREC5512 treated with 1 × and 2 × MIC of tigecycline resumed normal growth after 8 hours. However, the two isolates treated at 4 × MIC of tigecycline were well inhibited (Figure 2). This suggests that we are prone to treatment failure when we apply insufficient starting drug concentrations and should pay more attention to drug doses in clinical settings. Moreover, it was also clearly observed in the time-kill curves that the bactericidal effect of eravacycline was stronger than that of tigecycline for four different types of strains (Figure 2). In Table 4, we identified 24 tigecycline-resistant isolates, including 3 *E. coli* isolates, 5 *K. pneumoniae* isolates, 2 *E. cloacae* complex isolates, and 14 *A. baumannii* isolates. We observed that tigecycline showed poor in vitro antibacterial activity against *A. baumannii* relative to other bacteria. Interestingly, the results also showed that 90% of tigecycline-resistant isolates were equally resistant to eravacycline. This phenomenon may be attributed to the presence of resistance genes and efflux pumps.^{36–40} In addition, comparing the drug sensitivity results of *K. pneumoniae* and *E. coli* in Table 4 showed that *K. pneumoniae* was less susceptible to eravacycline and tigecycline than *E. coli*. This was also

demonstrated in the time-kill curves (Figure 2). In brief, the time-kill curves showed that eravacycline had better bactericidal activity and a longer drug half-life against *Enterobacteriaceae* and *A. baumannii* than tigecycline, further indicating that eravacycline is promising for clinical application and may be an important option for the treatment of CRGNB infections.

Notably, our study has some potential limitations. Our studies are based on in vitro evidence of antimicrobial activity; therefore, the effect of application in humans could not be determined, and it is not known whether there is an effect in humans. The number of cases and specimens is not representative of the whole population, and more studies are needed to more fully evaluate the antibacterial activity of eravacycline.

Conclusions

In this study, we demonstrated that eravacycline exhibited a high antibiotic potency on CRGNB, especially on CRKP and CREC. In addition, we observed that CRGNB showed an increasing trend in MIC values for eravacycline and tigecycline, and carried carbapenem resistance genes such as *bla*_{KPC} and *bla*_{NDM}. Therefore, eravacycline usage in clinics should receive more surveillance in its susceptibility.

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki and was reviewed and approved by the Research Ethics Committee of Zhejiang Provincial People's Hospital (QT2022307).

Acknowledgments

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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