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In vitro activity of eravacycline against common ribotypes of *Clostridioides difficile*

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Background: Eravacycline is a novel synthetic fluorocycline antibacterial approved for complicated intra-abdominal infections.

Objectives: The purpose of this study was to assess the *in vitro* activities of eravacycline and comparator antibiotics against contemporary clinical isolates of *Clostridioides difficile* representing common ribotypes, including isolates with decreased susceptibility to metronidazole and vancomycin.

Methods: Clinical *C. difficile* strains from six common or emerging ribotypes were used to test the *in vitro* activities of eravacycline and comparator antibiotics (fidaxomicin, vancomycin and metronidazole) by broth microdilution. In addition, MBC experiments, time–kill kinetic studies and WGS experiments were performed.

Results: A total of 234 isolates were tested, including ribotypes RT001 ($n=37$), RT002 ($n=41$), RT014-020 ($n=39$), RT027 ($n=42$), RT106 ($n=38$) and RT255 ($n=37$). $MIC_{50/90}$ values were lowest for eravacycline ($\leq$0.0078/0.016 mg/L), followed by fidaxomicin (0.016/0.063 mg/L), metronidazole (0.25/1.0 mg/L) and vancomycin (2.0/4.0 mg/L). MBCs were lower for eravacycline compared with vancomycin for all ribotypes tested. Both vancomycin and eravacycline demonstrated bactericidal killing, including for epidemic RT027. The presence of the *tetM* or *tetW* resistance genes did not affect the MIC of eravacycline.

Conclusions: This study demonstrated potent *in vitro* activity of eravacycline against a large collection of clinical *C. difficile* strains that was not affected by ribotype, susceptibility to vancomycin or the presence of certain *tet* resistance genes. Further development of eravacycline as an antibiotic to be used in patients with *Clostridioides difficile* infection is warranted.

Introduction

Clostridioides difficile infection (CDI) is the most common healthcare-associated infection in the USA with approximately 500000 cases annually.[1,2] CDI is generally treated with oral antibiotics; however, in cases of fulminant CDI, IV antibiotics are recommended.[3] IV antibiotics may also be given if the patient is unable to tolerate oral medications. Historically, IV metronidazole has been the antibiotic of choice in these cases; however, due to declining efficacy, oral metronidazole is no longer guideline recommended for mild–moderate or severe CDI.[4] Unfortunately, there is a lack of other evidence-based IV options for CDI and IV metronidazole continues to be recommended for fulminant CDI. Thus, there is an urgent unmet medical need to identify an IV antibiotic with *in vitro* and pharmacological activity against *C. difficile*.

Eravacycline is a novel synthetic fluorocycline antibacterial that was FDA approved for complicated intra-abdominal infections in 2018.[5] In Phase III clinical trials, no cases of CDI were observed.[5,6] Likewise, tigecycline and other tetracyclines display *in vitro* activity against *C. difficile*.[7,8] Two studies have investigated the effect of eravacycline against anaerobes, including *C. difficile*; however, they did not perform strain typing or focus on antibiotic-resistant

strains.[9,10] The purpose of this study was to assess the *in vitro* activities of eravacycline and comparator antibiotics against contemporary clinical *C. difficile* isolates representing common ribotypes, including isolates with decreased susceptibility to metronidazole and vancomycin.

Methods

Ethics

Isolates were obtained from our ongoing, multicentre clinical study of patients with CDI hospitalized in two large health systems (13 hospitals in total) in the Houston, TX, area.[11] The ongoing study is approved by the University of Houston Committee for the Protection of Human Subjects with a waiver of informed consent (IRB study 00000128). A randomly chosen, convenience sample of isolates from patients ≥18 years of age with CDI who had specimen ribotype data available was selected for this study.

Microbiology and C. difficile identification

Cryofrozen isolates were enriched overnight at 37°C in brain heart infusion (BHI) broth with oxyrase under anaerobic conditions. Overnight cultured isolates were streaked on cycloserine cefoxitin fructose agar (CCFA) plates and incubated under anaerobic conditions for 48 h. Isolates were confirmed to be *C. difficile* on the basis of Gram stain results, typical odour and the presence of *C. difficile* antigen on Microscreen latex agglutination (Microgen Bioproducts Ltd, Surrey, UK). Fluorescent PCR ribotyping was performed as previously described.[12,13] For this study, clinical strains from the six most common or emerging ribotypes in our collection were used: RT001, RT002, RT014-020, RT027, RT106 and RT255.[14]

Antimicrobials

Eravacycline was provided by the sponsor (Tetraphase Pharmaceuticals, Inc., Watertown, MA, USA). Metronidazole, fidaxomicin and vancomycin were purchased from Sigma–Aldrich, Inc. (St Louis, MO, USA).

In vitro susceptibility

In vitro susceptibility of the clinical strains of *C. difficile* to eravacycline and comparator antibiotics (fidaxomicin, vancomycin and metronidazole) was assessed using the broth microdilution method as previously described.[15] MIC panels containing 2-fold dilutions of eravacycline and comparators (range = 0.03–16 mg/L) in supplemented BHI broth were prepared. Fidaxomicin was diluted in DMSO and further diluted with distilled water to each final concentration. Each isolate was streaked onto a blood agar plate and incubated overnight. A single isolated colony from the blood agar plate was suspended in BHI/Mueller–Hinton broth supplemented with vitamin K and 5 μg/mL haemin to achieve a turbidity equal to that of a 0.5 McFarland standard. One hundred microlitres of the suspension was added to microtitre wells for a final concentration of $\sim 1\times10^6$ cfu/mL. The MIC was defined as the lowest concentration of the agent that inhibited growth at 24 h. Reference strains (*Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741 and *C. difficile* ATCC 700059) were included as controls. All assays were performed at least in duplicate.

MBC assay

One isolate from each ribotype was further assessed for MBC determination. Following incubation and analysis of the MIC plates, 10 μL aliquots from the MIC well and three wells above the MIC were spotted onto the surface of pre-reduced *Brucella* agar supplemented with 5% sheep blood and vitamin K1 (1 mg/L) to determine the MBC in accordance with CLSI guidelines.[16] Plates were incubated anaerobically overnight at 37°C. The highest dilution that yielded no single colony was considered the MBC.

Time–kill kinetic studies

Cultures were prepared from one isolate of each *C. difficile* ribotype by inoculating 20 mL of BHI-supplemented broth with a single colony of each ribotype. Cultures were grown for approximately 18 h to achieve a turbidity equal to that of a 0.5 McFarland standard. One hundred microlitres of the suspension was added to microtitre wells for a final concentration of $\sim 1\times10^6$ cfu/mL. Eravacycline at 8×, 16× or 32× the MIC was added along with negative controls. Total viable counts were determined immediately (time 0) and at 24 and 48 h post-inoculation. Samples were withdrawn at each timepoint, centrifuged (1 min at 16 000 **g**) and washed twice in sterile pre-reduced PBS to reduce residual drug carry-over, before 10-fold serial dilutions were performed prior to plating on BHI-supplemented agar. Agar plates were incubated for 24 h, following which the number of viable *C. difficile* (cfu/mL) was determined. The limit of detection for killing kinetic assays was 50 cfu/mL. Bactericidal activity was defined as a reduction of ≥3 $\log_{10}$ in viability relative to the starting inoculum after 24 h of exposure to antibiotics.

Table 1. MIC distributions of eravacycline and comparators for 234 strains of *C. difficile*

Ribotype	Sample size	MIC (mg/L)	Eravacycline	Fidaxomicin	Metronidazole	Vancomycin
Total	234	MIC_{50}	≤0.0078	0.016	0.25	2
		MIC_{90}	0.016	0.063	1	4
RT001	37	MIC_{50}	≤0.0078	0.016	0.25	2
		MIC_{90}	0.016	0.063	1	4
RT002	41	MIC_{50}	≤0.0078	0.016	0.25	2
		MIC_{90}	0.016	0.063	1	4
RT014-020	39	MIC_{50}	≤0.0078	≤0.016	0.125	2
		MIC_{90}	0.016	0.0635	1	2
RT027	42	MIC_{50}	≤0.0078	0.03	0.25	2
		MIC_{90}	0.13	0.063	0.5	4
RT106	38	MIC_{50}	≤0.0078	0.016	0.25	2
		MIC_{90}	0.03	0.063	1	4
RT255	37	MIC_{50}	≤0.0078	0.016	0.25	2
		MIC_{90}	0.13	0.063	0.5	4

Table 2. Eravacycline MIC stratified by susceptibility to vancomycin or metronidazole

	Eravacycline	
	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Vancomycin		
MIC <1 mg/L (n = 25)	0.001	0.008
MIC 1–2 mg/L (n = 157)	0.001	0.008
MIC >2 mg/L (n = 52)	0.001	0.03125
Metronidazole		
MIC <1 mg/L (n = 208)	0.001	0.008
MIC ≥1 mg/L (n = 26)	0.008	0.125

Table 3. Eravacycline and vancomycin MIC and MBC values by ribotype (n = 1, each)

	Vancomycin		Eravacycline	
Ribotype	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)
RT001	0.5	1	<0.0078	0.0315
RT002	0.5	2	<0.0078	<0.0315
RT014-020	0.5	0.5	<0.0078	0.015
RT016	1	4	<0.0078	0.0078
RT027	0.25	0.5	<0.0078	0.0078
RT255	0.25	1	<0.0078	0.015

WGS and resistance gene determinants

A convenience sample of isolates from six distinct ribotypes underwent DNA extraction using either the QIAamp DNA Mini Kit (QIAGEN, Venlo, the Netherlands) or the AnaPrep automated DNA extractor (BioChain Institute Inc., Newark, CA, USA) as previously described.[17] DNA was quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit (Thermo Fisher Scientific, Waltham, MA, USA) and DNA quality was assessed using a BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). DNA libraries were prepared according to Illumina's protocols, multiplexed on a flow cell and run on a NextSeq (Illumina Inc., San Diego, CA, USA) using paired-end sequencing. Sequence data were mapped against the *C. difficile* 630 reference genome as previously described.[18] Sequences were compared using SNPs, obtaining differences between sequences from maximum likelihood phylogenies constructed from mapped read data using PhyML version 3.1[19] (with generalized time-reversible substitution model and 'BEST' tree topology search algorithm), and corrected for recombination using ClonalFrameML version 1.25[20] (with default settings). Sequence reads were also *de novo* assembled with Velvet[21] using the Velvet optimizer; BLAST searches were used to identify the presence of resistance genes, including *tetM*, *tetO*, *tetW*, *tetO/32/O*, *tetB(P)*, *tet40*, *tetA(P)* and *tetL* as in Dingle *et al.*[22] and also *tetX* using an e-value for matches of 0.01. All matches were considered, including if spanning multiple contigs. Where present all matches covered ≥95% of the respective *tet* genes. Genes were matched to core genome MLST (cgMLST) using the database available at cgMLST.org.

Results

In vitro susceptibility

A total of 234 isolates were tested, including ribotypes RT001 (n = 37), RT002 (n = 41), RT014-020 (n = 39), RT027 (n = 42), RT106 (n = 38) and RT255 (n = 37). MIC_{50} values were lowest for eravacycline (≤0.0078 mg/L), followed by fidaxomicin (0.016 mg/L), metronidazole (0.25 mg/L) and vancomycin (2.0 mg/L). MIC_{90} values were also lowest for eravacycline (0.016 mg/L), followed by fidaxomicin (0.063 mg/L), metronidazole (1.0 mg/L) and vancomycin (4.0 mg/L). A summary of susceptibility results by ribotype is shown in Table 1. Eravacycline displayed potent activity against all

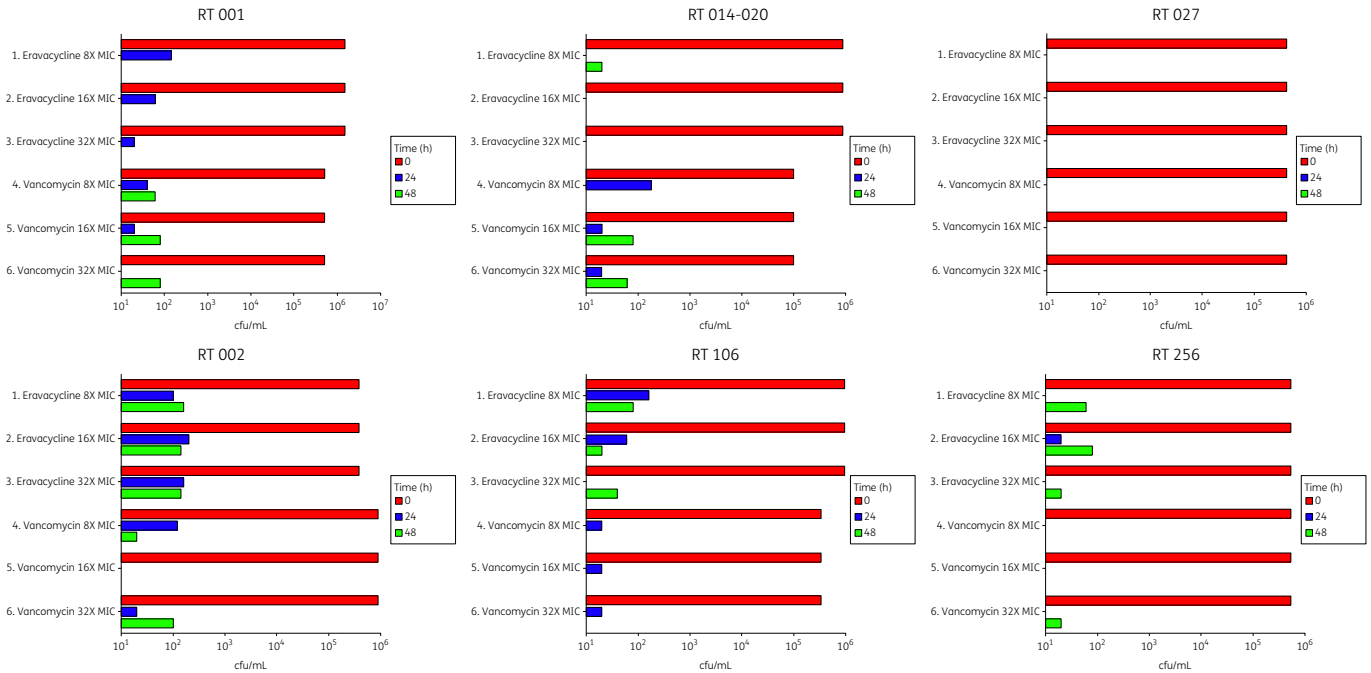

Figure 1. Time–kill experiments by ribotype, drug and MIC.

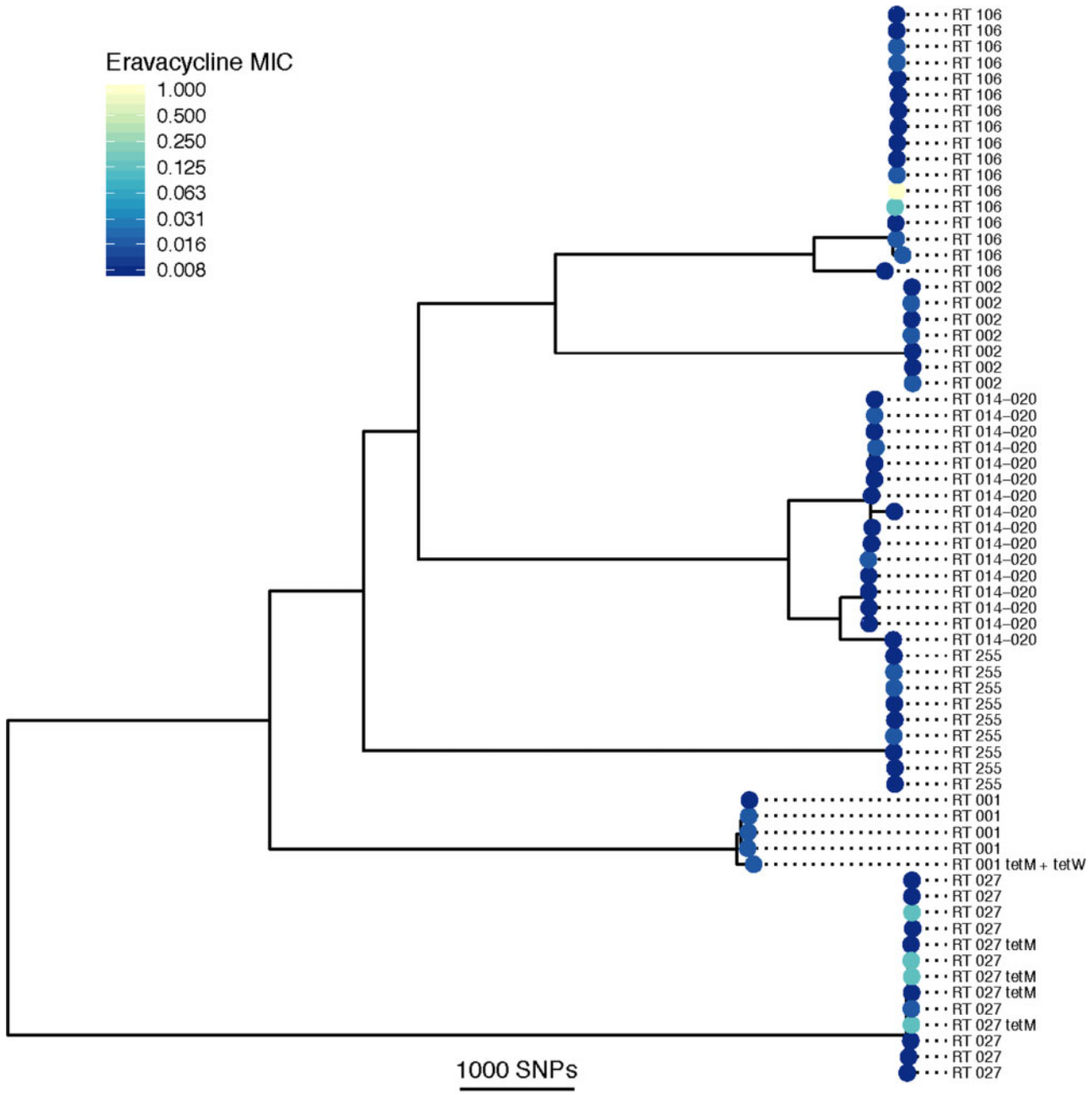

Figure 2. Phylogram of *C. difficile* isolates and *tet* resistance genes.

C. difficile strains regardless of ribotype. Decreasing susceptibility to vancomycin (MIC <1, 1–2 or >2 mg/L) had a minimal effect on the MIC_{50} (0.001 mg/L) or the MIC_{90} (0.008–0.03 mg/L) of eravacycline. However, eravacycline MIC_{50} and MIC_{90} values did increase with increasing MIC values of metronidazole (Table 2).

MBCs and time–kill kinetics

MBCs by ribotype are shown in Table 3. MBCs were lower for eravacycline compared with vancomycin for all ribotypes tested (eravacycline MBC range <0.03–0.015). Both vancomycin and eravacycline demonstrated bactericidal killing at 8×, 16× and 32× the MIC. Bactericidal killing was observed for all ribotypes, including epidemic RT027 (Figure 1).

WGS and resistant determinants

WGS data were available for 67 isolates, including RT106 ($n = 17$), RT002 ($n = 7$), RT014-020 ($n = 16$), RT255 ($n = 9$), RT001 ($n = 5$) and RT027 ($n = 13$). The most common cgMLST types corresponding to each ribotype were MLST3 (RT001), MLST8 (RT002), MLST2 (RT014-020), MLST1 (RT027), MLST42 (RT106) and MLST34 (RT255). *tet* resistance genes were identified in five isolates, including four RT027 isolates

with *tetM* and one RT001 isolate with *tetM* and *tetW*. The presence of a *tet* resistance gene did not affect the MIC of eravacycline (Figure 2).

Discussion

CDI is generally treated with the oral antibiotics vancomycin or fidaxomicin as they are non-absorbable and achieve high colonic concentrations.[3] However, in patients with fulminant CDI, IV antibiotics are guideline recommended to assure adequate colonic concentrations of an effective antibiotic. *C. difficile* displays *in vitro* susceptibility to tetracyclines and several case series have shown another tetracycline, i.e. tigecycline, to be clinically effective for CDI.[7,23] However, the adverse events of tigecycline, including high rates of nausea and vomiting and an FDA Black Box warning of increased risk of death, limit its use. Eravacycline is available in an IV formulation and is primarily excreted via the faeces in its active form, making it a potential option for the treatment of CDI.[24] Two previous small-scale studies evaluated the activity of eravacycline against *C. difficile* isolates; however, neither had a specific focus on *C. difficile* or tested a broad range of different *C. difficile* ribotypes.[6,7] In these previous studies, eravacycline $MIC_{50/90}$ was 0.12/1 and 0.06/0.13 mg/L, consistent with our current study ($\leq$0.0078/0.016 mg/L). These previous studies used the CLSI-recommended agar dilution method, which can produce higher MICs than the broth microdilution used in this study.[15] Minimal differences in $MIC_{50/90}$ were observed between ribotypes or in isolates with elevated MICs of vancomycin. As expected, the presence of *tetM* or *tetW* resistance genes did not affect the MIC of eravacycline.[25] An increased eravacycline MIC was observed with increased metronidazole MIC; this effect will need to be confirmed in future studies. The MBC of eravacycline was within two to three 2-fold dilutions of the MIC. Time–kill kinetic studies confirmed a bactericidal effect similar to vancomycin. Future studies on multiple strains of each ribotype will be needed to confirm these results. Taken together, these experiments provide strong *in vitro* evidence for the further development of eravacycline as a treatment option for CDI. The effect of eravacycline on the microbiome should be evaluated with *in vitro* CDI gut models and *in vivo* animal trials. In addition, because of eravacycline's broad spectrum of activity, it is possible that eravacycline could reduce the colonization of certain MDR organisms as well.[6] The effect of eravacycline on these pathobionts as well as normal microbiota should be evaluated.

In conclusion, this study demonstrated the potent *in vitro* activity of eravacycline against a large collection of *C. difficile* strains. The MIC and MBC of eravacycline were not affected by *C. difficile* ribotype, susceptibility to vancomycin or the presence of certain *tet* resistance genes. Further development of eravacycline as a treatment option for CDI is warranted.

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Transparency declarations

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