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Highlights

- Excellent activity of eravacycline against enterocci including VRE isolates
- First description of enterococcal clinical isolates resistant to eravacycline
- Resistance to eravacycline is associated with resistance to all tetracyclines
- Eravacycline resistant isolates have at least two *tet* genes and one *rpsJ* mutation

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In vitro activity of eravacycline and mechanisms of resistance in enterococci

Running title: Eravacycline resistance in enterococci

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Abstract

Eravacycline (ERC), the first fluorocycline, is a new tetracycline with superior activity to tigecycline (TGC) against many bacterial species. The objective of this work is to determine the *in vitro* activity of ERC and compare to other tetracyclines against enterococcal clinical isolates and to analyze corresponding resistance mechanisms. A collection of 60 strains of enterococci was studied: 54 epidemiologically unrelated clinical isolates (46 *Enterococcus faecium* and 8 *Enterococcus faecalis*) including 42 vancomycin resistant enterococci (VRE) (33 *vanA* and 9 *vanB*), 3 *in vitro* TGC-resistant mutants (*E. faecium* AusTig, HMtig1 and HMtig2) and 3 reference wild-type strains (*E. faecium* Aus0004 and HM1070, *E. faecalis* ATCC 29212). *In vitro* susceptibility was determined using E-test strips (ERC) or by broth microdilution (TGC, doxycycline [DOX], minocycline [MIN], tetracycline [TET]). Resistance genes (*tet*(M), *tet*(L), *tet*(O) and *tet*(S)) were screened by PCR for TGC- and/or ERC-resistant strains as well as sequencing of the *rpsJ* gene (coding for the S10 ribosomal protein). $MIC_{50/90}$ values were 0.01/0.12, 0.03/0.5, 4/32, 8/16 and 32/>32 mg/L for ERC, TGC, DOX, MIN and TET, respectively. According to EUCAST guidelines, 9 strains were categorized as resistant to TGC (MIC=0.5-8 mg/L), including 4 strains of *E. faecium vanA*(+) also resistant to ERC (MIC=0.25-2 mg/L). These 4 strains all possessed at least 1 mutation in *rpsJ* and two *tet* determinants: *tet*(M) + *tet*(L) (n=2), *tet*(M) + *tet*(S) (n=2). Although ERC has an excellent *in vitro* activity against enterococci (including VRE), emergence of resistance is possible, due to combined mechanisms (*rpsJ* mutations + *tet* genes).

Keywords: *Enterococcus*; VRE; Eravacycline ; Tigecycline; Tetracycline resistance.

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1. Introduction

Enterococci, in particular *E. faecalis* and *E. faecium*, are opportunistic pathogens that are increasingly responsible for hospital-acquired infections and acquisition of multidrug resistance (MDR) is an important issue for therapeutic management [1]. Numerous clinical isolates, with regionally varying incidence, have especially acquired resistance to vancomycin (vancomycin-resistant enterococci [VRE]) [2]. For the treatment of VRE infections, only a few therapy options remain available such as linezolid, daptomycin or tigecycline (TGC) [1].

As a member of the tetracycline family, TGC (a semi-synthetic derivative of minocycline) acts by inhibiting bacterial protein biosynthesis by binding to the 30S subunit of the ribosome. Resistance to classical tetracyclines is mainly due to ribosomal protection and/or active efflux pump. Different classes of ribosomal protection proteins exist, the most common ones being encoded by *tet*(M), *tet*(O) and *tet*(S) genes. Several efflux pump-encoding genes have been described both in Gram-positives [e.g. *tet*(K) and *tet*(L)] and Gram-negatives [e.g. *tet*(A) and *tet*(B)] [3]. All these genes are usually located on plasmids and are then transferable. In enterococci, the most frequent genes implicated in tetracycline resistance are *tet*(L), *tet*(M), *tet*(O) and *tet*(S) while ribosomal protection is the most common acquired mechanism of resistance [4, 5]. Interestingly, TGC is not or only poorly affected by these classical tetracycline resistance determinants and the mechanism of TGC decreased susceptibility in enterococci is associated with alterations of the S10 protein of the ribosomal 30S subunit encoded by the *rpsJ* gene or the overexpression of *tet*(M) and *tet*(L) genes [6–9]. Even though enterococcal clinical isolates resistant to TGC have been mainly reported sporadically, some hospital outbreaks due to TGC-resistant epidemic clones have been described in Brazil [8] and Germany [9], highlighting their potential risk of spread.

Novel compounds of the tetracycline family, including eravacycline (ERC) have been recently developed. ERC, formerly known as TP-434, is a fully synthetic fluorocycline developed to escape the resistance mechanisms of the tetracycline class as efflux pumps and ribosomal protection mechanisms. Its possible oral use makes it more advantageous than tigecycline. Intravenous

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administration is also possible, in this case the serum concentrations obtained are higher than those obtained with tigecycline. In addition, the MIC to the ERC of the different strains tested are significantly lower than those of TGC, making ERC a promising antibiotic [10–12]. This molecule acts like the other tetracyclines by reversibly binding to the ribosomal 30S subunit and preventing the bacterial protein synthesis [3]. It has excellent activity *in vitro* and *in vivo* on both Gram-positive and Gram-negative bacteria and anaerobes, including MDR strains such as VRE [13-15]. Its use is recommended by the FDA in August 2018 for complicated abdominal infections [16].Some ERC-resistant clinical isolates of enterobacteria, *A. baumannii* and *S. aureus* have been reported in the literature [17-19], but no ERC-resistant enterococci have been described so far.

The aim of the work was first to study the *in vitro* activity of ERC against clinical isolates enterococci including VRE and to compare it to other tetracyclines. Secondly, an analysis of the associated resistance mechanisms was performed.

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2. Materials and Methods

2.1. Bacterial isolates

A panel of 60 non-duplicate strains of enterococci was studied consisting of: 54 epidemiologically-unrelated clinical isolates (46 *E. faecium* and 8 *E. faecalis*) including 42 VRE (33 *vanA* positive and 9 *vanB* positive), 3 *in vitro* mutants resistant to TGC (*E. faecium* AusTig, HMtig1 and HMtig2 [6]) and 3 reference strains (*E. faecium* Aus0004 [20] and HM1070 [21], *E. faecalis* ATCC 29212). All the clinical TGC-resistant isolates received at the National Reference Centre for Enterococci (university hospital of Rennes, France) from all French hospitals between 1st January 2017 and 31st December 2018 were included (as positive controls) as well as TGC-susceptible epidemiologically-unrelated strains (as negative controls). They were isolated from rectal swabs (n=39), urines (n=7), blood cultures (n=4) and surgical samples (n=4). All the strains were identified by MALDI-TOF mass spectrometry (Microflex; Bruker Daltonics, Bremen, Germany).

2.2. Antimicrobial susceptibility testing

The MICs of the different tetracyclines tested (ERC, TGC, doxycycline [DOX], minocycline [MIN] and tetracycline [TET]) were determined by broth microdilution (BMD) method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [22], except for ERC (MIC test strips supplied by Liofilchem; Roseto degli Abruzzi, Italy). For BMD, the serialdilution of antibiotics (all prepared extemporaneously) were from 32 to 0.03 mg/L and the MH medium used (Becton Dickinson, Spark, MD, USA) was adjusted to provide 20 to 25 mg/L of calcium and 10 to 12.5 mg/L of magnesium. The MIC is defined as the lowest concentration of antimicrobial agent that inhibited the growth at 35±2°C after 24 h of incubation in aerobic conditions. Results were determined and interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints for TGC (sensitive (S) ≤ 0.25 mg/L; resistance (R) > 0.25 mg/L) and ERC (S ≤ 0.125 mg/L; R > 0.125 mg/L) (www.eucast.org). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 served as quality control strains for each tested batch.

2.3. Gene resistance screening

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Genomic DNA of TGC- and ERC-resistant strains was extracted using the InstaGene kit (Bio-Rad, Hercules, CA). The *tet*(L), *tet*(M), *tet*(O) and *tet*(S) genes were screened, and *rpsJ* gene was amplified by performing specific real-time PCR assays (primers and probes previously described by Cattoir et *al.*[6]). All *rpsJ* PCR-amplified products were sequenced in both directions by the Sanger method using the same primers . RpsJ deduced-amino-acid sequences obtained thanks to the alignment tool of CLC Genomics Workbench (Qiagen) were compared with those of reference strains *E. faecium* DO (GenBank accession no. NC_017960) and *E. faecalis* V583 (GenBank accession no. NC_004668) by using the BLASTX program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The screening for vancomycin resistance genes was also done by qPCR as previously described [23].

3. Results

3.1. In vitro activity of tetracyclines against enterococci

Of the different tetracyclines tested, ERC showed the lowest MIC_{90} (0.08 mg/L) as compared to the other tetracyclines (Table 1). MIC_{50} was higher for *E. faecalis* strains (between 1 and 5-fold-dilutions apart) compared to *E. faecium* strains. MIC_{90} were higher for *E. faecium* isolates for ERC (0.08 mg/L) and TGC (0.5 mg/L) with one and two-fold-dilutions difference respectively. MIC_{90} was identical between the two species for the other tetracyclines.

In *E. faecium* strains, MIC_{50} and MIC_{90} values were identical for ERC and TGC regardless of the susceptibility to vancomycin whereas for the other molecules a higher value of at least one fold-dilution was observed in VRE strains (Table 1). Focusing on VRE strains, a significant difference was observed between strains carrying the *vanA* and *vanB* genes (Table 1). Indeed, MIC_{90} values of ERC and TGC were 0.19 mg/L and 1 mg/L for VRE *vanA*(+) versus 0.016 mg/L and 0.06 mg/L for *vanB*(+), respectively.

Since a critical concentration is only available for TGC and ERC, only results for these molecules were interpreted for susceptibility and resistance. Among the strains studied, 8 *E. faecium* and 1 *E. faecalis* were categorized as resistant to TGC with MICs ranging from 0.5 to 8 mg/L, MICs of classical

tetracyclines being variable (Table 2). Four of the TGC-R (TGC MICs ≥1mg/L) strains were also resistant to ERC with MICs between 0.19 and 1.5 mg/L, and also exhibited high MICs for all the classical tetracyclines (Table 2).

3.2. Mechanisms of resistance to TGC and/or ERC

To decipher the mechanisms of resistance to TGC and ERC, *tet* genes were screened in resistant strains. Most of the strains possessed one or two *tet* resistance genes: *tet*(L) (n=1), *tet*(M) (n=2), *tet*(L) + *tet*(M) (n=3) and *tet*(M) + *tet*(S) (n=2) (Table 2). None *tet*(O) was detected. None of these resistance genes were been found in the *E. faecalis* isolate and *in vitro* mutants HMtig1 and HMtig2, which was expected since the parental strain HM1070 is susceptible to tetracyclines [6].

Note that all the ERC-R isolates (n=4) carried the *tet*(M) gene, two of them also carried the *tet*(L) gene and the other also *tet*(S) gene (Table 2). All strains resistant to ERC were strains of *E. faecium* resistant to vancomycin (*vanA* positive).

The amino acid sequence of the S10 protein (encoded by *rpsJ)* was also determined in ERC- and/or TGC-resistant strains. Two types of modifications were observed, substitutions (most frequently) and deletions, all between amino acids 52 and 60 (Table 2). All strains resistant to ERC and TGC (n=4) displayed one non-synonymous substitution: $I_{52}M$ (n=1), $H_{56}N$ (n=2) and $K_{57}R$ (n=1). Three of these strains additionally had a deletion of two (delR53_A54, n=1) or four amino acids (delI52_T55, n=2). Concerning the TGC-resistant/ERC-susceptible strains (n=5), all *E. faecium* strains had a protein S10 substitution: $D_{60}Y$ (n=4) and $K_{57}E$ (n=1). The *E. faecalis* strain did not show any *rpsJ* mutation.

The more strains accumulated *tet* genes and S10 protein modification, the higher their tetracycline MICs were. All ERC-R strains had two *tet* resistance genes and one S10 protein alteration. *E. faecium* strains TGC-R and ERC-S had one *tet* resistance gene and/or a mutation on *rpsJ* gene.

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4. Discussion

ERC has shown good *in vitro* activity against a wide panel of bacteria in many studies [14-15], we find similar results with a low MIC_{90} at 0.08 mg/L against enterococci including VRE. In this study, we report for the first time four ERC-resistant clinical enterococcal isolates, which is in line with resistance previously reported in Gram-negative (overexpression of efflux pumps and tetracycline-inactivating enzyme) [18, 19, 24] and other Gram-positive (efflux pumps and ribosomal protection) [17] bacteria.

Analysis of these four strains showed that the occurrence of ERC resistance resulted from an accumulation of several resistance mechanisms. Indeed, these strains exhibited high MICs for all the tetracyclines studied, suggesting a high-level resistance to this class of antibiotics. Moreover, for each of them, two *tet* resistance genes were simultaneously found, i.e. *tet*(M) + *tet*(L) or *tet*(M) + *tet*(S). These acquired resistance genes were associated with alterations in the S10 protein in all cases. All these elements suggest that resistance to ERC in enterococci would require the presence of at least two *tet* genes associated with an *rpsJ* mutation. The ERC was initially developed to deal with classical acquired resistance mechanisms (active efflux and ribosomal protection) and numerous studies have shown its effectiveness against strains carrying them, including the very common Tn*916*-like-associated *tet*(M) gene [3, 10–12, 25]. All ERC-R enterococcal isolates studied were strains of *E. faecium vanA*(+) suggesting plasmid acquisition of previously reported *tet* genes as described by others [26]; different expression of the *tet* genes (plasmid versus. chromosome) depending on the strain studied could explain the absence of an *in vitro* ERC-R mutant.

All ERC-resistant isolates were also highly resistant to TGC with high MICs (≥1 mg/L) suggesting the evolutionary potential of these enterococcal strains and the risk of therapeutic failure by ERC in case of infections caused by strains highly resistant to TGC. No significant difference was observed between the ERC and TGC MIC_{90} of VRE and vancomycin-susceptible strains, which may be surprising since MIC_{90} reflect acquired resistance, it could be explained by the low number of vancomycin-susceptible strains tested (8 vs. 43).

Focusing on the S10 protein, the location of the *rpsJ* changes seems to be important and responsible for different resistance levels. Indeed, alterations located between amino acids 57 and 60 seem to be responsible for resistance to TGC as described above [6, 27] and not to ERC. The alterations of the amino acids between positions 54 and 57 would lead to resistance to both TGC and ERC, which has not yet been described to our knowledge and remains to be demonstrated experimentally. Changes in these amino acids are probably significant enough to affect the conformation of the 16S rRNA and therefore prevent both TGC and ERC from binding to the ribosome. For the TGC-resistant/ERC-susceptible strains, a single *tet* gene and a single *rpsJ* mutation (non-synonymous substitution) were found. Based on our results, deletions of *rpsJ* in enterococci seem to be necessary to confer ERC resistance.

One strain of TGC-resistant *E. faecalis* was found in our panel. Neither the presence of a *tet* gene nor a *rpsJ* modification were identified suggesting that the involvement of other mechanisms such as another *tet* gene or chromosomal mutation, or less likely a Tet(X)-mediated enzymatic inactivation [4].

5. Conclusion

It seems difficult to predict the evolution of tetracycline resistance, in particular through the evolution of *rpsJ* gene mutations *in vivo* in exposed patients, demonstrating the non-negligible risk of selection of tetracycline-resistant mutants [28]. Although having an excellent *in vitro* activity, ERC does not escape the resistance mechanisms already reported as TGC.

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Declarations

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Competing Interests: No

Ethical Approval; Not required

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Table 1. *In vitro* activity (MICs in mg/L) of tetracyclines against enterococci

Strains	Eravacycline			Tigecycline			Doxycycline			Minocycline			Tetracycline		
	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range
All (n=60)	0.016	0.08	0.004-1.5	≤0.03	0.5	≤0.03-8	4	32	≤0.03->32	8	16	≤0.03-32	32	>32	≤0.03->32
E. faecium (n=51)	0.016	0.08	0.004-1.5	≤0.03	0.5	≤0.03-8	4	32	≤0.03->32	0.5	16	≤0.03-32	16	>32	0.06->32
vancomycin-susceptible (n=8)	0.016	0.08	0.008-0.08	≤0.03	0.5	≤0.03-0.5	0.125	4	≤0.03->32	0.125	8	≤0.03-32	0.25	32	0.125->32
vancomycin-resistant (n=43)	0.016	0.08	0.004-1.5	≤0.03	0.5	≤0.03-8	8	32	≤0.03->32	8	16	≤0.03-32	32	>32	0.06->32
vanA (n=32)	0.016	0.19	0.004-1.5	≤0.03	1	≤0.03-8	8	32	≤0.03->32	8	16	≤0.03-32	32	>32	0.06->32
vanB	0.012	0.016	0.008-	≤0.03	0.06	≤0.03-	0.125	16	≤0.03-	16	16	≤0.03-	0.25	32	0.06-

(n=11)			0.023			0.5			16			16			>32
E. faecalis (n=9)	0.023	0.032	0.008-0.032	0.06	0.125	≤0.03-0.5	16	32	0.06->32	16	16	≤0.03-16	>32	>32	≤0.03->32

MIC_{50}, MIC_{90} and MIC range of each tetracyclines tested are represented for all the isolates studied in the first line and then considering bacterial species and vancomycin-susceptibility.

Table 2. Characteristics of eravacycline- and/or tigecycline-resistant isolates and reference strains

	MIC (mg/L)[a]					Detection of *tet* genes				
Isolates	**ERC**	**TGC**	**DOX**	**MIN**	**TET**	***tet*(M)**	***tet*(L)**	***tet*(O)**	***tet*(S)**	**RpsJ alterations[b]**
E. faecium DO						-	-	-	-	42 47 52 57 62 67 7 LPTERSLYTIIRATHKYKDSR EQFEMRTHKRL
E. faecium 17-477	**1.5**	**8**	16	16	>32	**+**	**+**	-	-	**********: **********
E. faecium 18-481	**0.19**	**1**	16	32	>32	**+**	-	-	**+**	********** :**********
E. faecium 18-626	**0.19**	**1**	8	32	>32	**+**	-	-	**+**	********** :**********
E. faecium 18-785	**0.25**	**2**	>32	32	>32	**+**	**+**	-	-	**********:***** **********
E. faecium 18-394.1	0.047	**0.5**	>32	32	>32	-	**+**	-	-	**********:** **********
E. faec	0.023	**0.5**	1	0.5	0.5	**+**[c]	-	-	-	**********:** **********

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ium Aus Tig										
E. faecium HMtig1	0.023	**0.5**	2	0.5	0.5	-	-	-	-	**********:**********
E. faecium EF16	0.047	**0.5**	16	16	>32	**+**	-	-	-	**********:**********
E. faecium HMtig2	0.094	0.25[d]	2	0.25	2	-	-	-	-	**********:**********
E. faecium EF22	0.064	0.25[d]	4	0.5	16	+	+	-	-	********** **********
E. faecalis V583						-	-	-	-	LPTERSLYTVIRATHKYKDSR EQFEMRTHKRL
E. faecalis 18-106	0.023	**0.5**	0.06	≤0.03	≤0.03	-	-	-	-	********** **********

[a]ERC, eravacycline; TGC, tigecycline; DOX, doxycline; MIN, minocycline; TET, tetracycline. MIC of ERC and TGC are indicated in bold for resistant strains.

[b]*rpsJ* sequences were aligned against those of susceptible reference strains *E. faecium* DO and *E. faecalis* V583. Non-synonymous substitutions are indicated by a colon and deletion by a space for each amino acid (the absence of modification is represented by an asterisk). None synonymous substitution was observed.

[c]non-functional truncated *tet*(M) gene.

[d]Strains categorized as susceptible to TGC but with elevated MIC.

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