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Highlights:

- The co-presence of Y58D mutation in *rpsJ* gene and *tet*(M)/*tet*(L) genes is linked to tigecycline and eravacycline resistance in ST80 VREfm clinical isolates.
- Phylogenetic analysis revealed clonal spread of tigecycline- and eravacycline-resistant - VREfm isolates.
- Genomic surveillance is essential to track emerging resistance in vancomycin-resistant Enterococci.

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The Y58D mutation in *rpsJ* gene is correlated with tigecycline and eravacycline combined resistance in ST80 vancomycin-resistant *Enterococcus faecium* isolates

Felice Valzano[a], Elisa Beccia[a], Gianfranco La Bella[a,b], Marianna Marangi[a], Fabio Arena[a,c]#

[a]Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

[b]Azienda Sanitaria Locale della Provincia di Foggia, Foggia, Italy

[c]Microbiology and Virology Unit, Policlinico Foggia, Foggia, Italy

#Corresponding author: Fabio Arena, Department of Clinical and Experimental Medicine, University of Foggia, Via Luigi Pinto - c/o Centro di Ricerche Biomediche, 71122 Foggia, Italy, fabio.arena@unifg.it, +390881338864

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Abstract

Objectives: To describe the phenotypic and genetic features of vancomycin-resistant *Enterococcus faecium* (VREfm) isolates exhibiting resistance to tetracycline (TET), tigecycline (TGC) and eravacycline (ERV).

Methods: Between December 2023 and September 2024, 32 vancomycin-resistant Enterococci (VRE) were collected from clinical samples at the Policlinico Foggia, Italy. Bacterial identification was carried out by mass spectrometry analysis (MALDI-TOF). Antimicrobial susceptibility testing for vancomycin (VA), TET, TGC and ERV was performed by reference broth microdilution method. Whole-genome sequencing (WGS) was carried out to explore the content of tetracyclines resistance determinants, and the phylogenetic relationship between isolates (in silico MLST and SNPs analysis).

Results: Among studied isolates, thirty and two were *E. faecium* and *Enterococcus faecalis* (Efs), respectively. Overall, eight isolates, six VREfm and two VREfs, were resistant to TET (8/32, 25%), with four (all VREfm) being resistant to TGC and ERV (4/32, 12.5%), also. The two TET-resistant VREfs strains belonged to ST6 and carried the *tet*(M) gene only. All the TET-resistant VREfm were ST80 and carried *tet*(L) and *tet*(M) genes. Among these, the four TGC-resistant and ERV-resistant strains showed an uncommon Y58D substitution in the ribosomal S10 protein (*rpsJ* gene). SNPs analysis revealed that Y58D-carrying strains formed a separate cluster, within ST80 VREfm. Conclusions: In this study we correlated the presence of the Y58D mutation in the *rpsJ* gene with resistance to TGC and ERV in a cluster of VREfm ST80 clinical strains. The Y58D mutation was invariably found in co-presence with *tet*(L) and *tet*(M) genes.

Keywords: vancomycin-resistant *Enterococcus faecium*; tigecycline; eravacycline; *rpsJ* mutation; *tet* genes

1. Introduction

Enterococci are opportunistic pathogens that can cause life-threatening infections among hospitalized and immunocompromised patients [1]. The acquisition of genes associated with mobile genetic elements, such as the *van* clusters, led to the emerging of vancomycin-resistant Enterococci (VRE), which represent a serious problem in clinical settings. In Europe, a significantly increasing trend in the prevalence of vancomycin-resistant *Enterococcus faecium* (VREfm) bloodstream infections, over the last five years, has been reported [2]. The emergence of other determinants conferring resistance to non-glycopeptide drugs further limit treatment options for VRE-associated infections [1].

Tigecycline (TGC) (a glycylcycline deriving from the tetracyclines class of antibiotics) is a last-resort agent approved for the treatment of complicated skin and skin structure infections (excluding diabetes foot infection), complicated intra-abdominal infections, and community-acquired bacterial

pneumonia, in adults. TGC inhibits bacterial protein synthesis by binding the 30S ribosomal subunit [3]. TGC activity against Enterococci is not or only poorly affected by major classical tetracycline resistance determinants [i.e., efflux pumps such as Tet(L), and ribosomal protection proteins such as Tet(M) and Tet(O)]. By contrast, several studies have reported that mutations in the amino acid residues 53-60 of the S10 structural protein encoded by *rpsJ* gene (i.e., amino acid substitutions such as A54E, H56R, K57E, K57R, Y58S, D60Y, and deletions such as R53Q-Δ54-57ATHK) [4–8] can confer reduced susceptibility to TGC in both *E. faecium* and *Enterococcus faecalis* (Efs). Eravacycline (ERV), a synthetic halogenated tetracycline, was designed to overcome the most common resistance mechanisms to this class of antibiotics. ERV is a fluorocycline developed to treat infections caused by Gram-positive and Gram-negative organisms, including VRE [9]. However, recent evidence demonstrated that resistance mechanisms already described for TGC [4], as well as mutations in the 16S ribosomal RNA (e.g., G190A, G1058C, A926T, G927T) [10,11], can be also responsible for ERV resistance in Enterococci.

In this study we describe the phenotypic and genetic features of VREfm isolates exhibiting high level resistance to tetracycline (TET), TGC and ERV.

2. Material and methods

2.1. Isolates collection and bacterial identification

Between December 2023 and September 2024 at the Policlinico Foggia (Foggia, Southern Italy), 32 consecutive non-replicate VRE isolates from urine samples (n=20), blood cultures (n=7), surgical samples (n=3) and drainage fluids (n=2) were collected. Bacterial identification to the species level was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Billerica, MA, USA).

2.2. Antimicrobial susceptibility testing

Vancomycin (VA), TET, TGC and ERV susceptibility testing was performed in duplicate using reference broth microdilution method [12]. Results were interpreted according to the most recent clinical breakpoints from EUCAST (version 15.0, http://www.eucast.org) for VA, TGC and ERV, while CLSI breakpoints [13] were used for TET.

2.3. Whole-genome sequencing and genomic analysis

All collected isolates were subjected to whole-genome sequencing (WGS) experiments. A pre-extraction step was performed to optimize the DNA extraction. Briefly, overnight bacterial cultures were subjected to centrifugation at 5 000 *x* g for 10 minutes. The bacterial pellet was resuspended in 200 µL of 20 mg/mL lysozyme and incubated for 60 minutes at 37°C. Afterwards, bacterial DNA was extracted using an automated MagCore HF16 Plus System with the MagCore Genomic DNA Bacterial Kit (RBC Bioscience, Taipei, Taiwan), according to the manufacturer's recommendations. Genomic DNA was subjected to WGS on Illumina NovaSeq X Plus (Illumina Inc., San Diego, USA) platform, using a 2×150 bp paired-end reads approach.

Raw reads were subjected to the following filtering steps using fastp software (https://github.com/OpenGene/fastp): i) Illumina adapter removing, ii) removing of reads containing N>10% (i.e., reads with more than 10% uncertain nucleotides), and iii) removing of reads with more than 50% low quality nucleotides (Qscore≤5). The quality of sequence read data was checked using FastQC (https://github.com/s-andrews/FastQC). Subsequently, SPAdes v3.13.0 (https://github.com/ablab/spades) was used in the assembly mode "careful" with default parameters for de novo assembly of high-quality sequencing reads. Raw coverage of the assembled genomes ranged between 77.1× and 172.1×, with an average value of 104.4×. A mean of 558 contigs per bacterial genome was obtained, with an average N50 of 54 kbp.

Antimicrobial resistant genes were identified using ABRicate (https://github.com/tseemann/abricate), selecting NCBI AMRFinderPlus as reference database, while genetic variants of antimicrobial resistance-associated genes were detected using

pathogenwatch (https://github.com/pathogenwatch-oss). Mutations occurring in the *rpsJ* and 16S rRNA gene sequences were manually inspected using NCBI BLAST tools (https://blast.ncbi.nlm.nih.gov), selecting *E. faecium* SRR24 (GenBank accession no. **NZ_CP038996**) and *E. faecalis* EnGen0336 (GenBank accession no. **NZ_KB944666**) as reference genomes. In silico MLST analysis was performed by MLST software (https://github.com/tseemann/mlst). Phylogenetic relatedness between strains was investigated with the CSIPhylogeny 1.4 tool (https://cge.food.dtu.dk/services/CSIPhylogeny) using default parameters, except for the minimum distance between SNPs option which was disabled. The SNPs matrix and the phylogenetic tree were constructed using the VREfm ATCC 51559 genome (GenBank accession no. **JSVT00000000**) as reference. Phylogenetic tree was visualized and modified by FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

A schematic representation summarizing the study workflow is shown in Figure 1.

2.4. Data availability

Draft genome sequences were deposited in GenBank under the BioProject **PRJNA1227285**.

3. Results

All the 32 included VRE isolates were confirmed to be vancomycin-resistant (MIC >16 mg/L). Tetracyclines susceptibility testing showed that, out of 32 VRE isolates, eight (8/32, 25%) were resistant to TET (TET-R), showing a MIC >16 mg/L. Six out of the eight TET-R isolates were VREfm and two were VREfs. Among the six TET-R VREfm isolates, four were resistant to TGC (TGC-R) and ERV (ERV-R) (4/32, 12.5%), also, with a TGC MIC of 1 mg/L and an ERV MIC >0.5 mg/L (Table 1).

WGS analysis revealed that all strains carried the *vanA* gene cluster (*vanHAX*). Concerning tetracyclines resistance determinants, we found that the two TET-R VREfs strains carried the *tet*(M)

gene. All the TET-R VREfm carried *tet*(L) and *tet*(M) genes. Among these, the four TGC-R and ERV-R strains showed a non-synonymous amino acid substitution Y58D in the ribosomal S10 protein encoded by *rpsJ* gene (Table 1).

No other mutations at tetracyclines target sites in the 30S ribosomal subunit S10 (encoded by *rpsJ* gene) and 16S rRNA gene sequences known to be involved in the reduced susceptibility to TGC and/or ERV in Enterococci were detected in strains included in the study.

In silico MLST analysis showed that out of 30 VREfm strains, 15 belonged to sequence type (ST) 80, eight to ST117 and seven to the *pstS*-null ST1478. The VREfs strains belonged to ST6. Interestingly, all strains carrying Y58D substitution were VREfm belonging to ST80 (Table 1).

The SNPs analysis of the ST80 VREfm strains highlighted the presence of two main clusters: Cluster I including the four strains carrying both Y58D substitution in *rpsJ* gene and *tet*(L)/*tet*(M) gene combination and exhibiting a SNP range 102–580 [mean, 381 (SD, 160.1); median, 385.5 (IQR, 314–519)]; Cluster II including 10 strains with a wild-type *rpsJ* gene and exhibiting a SNP range 32–610 [mean, 193.6 (SD, 156.5); median, 150 (IQR, 60–241)] (Figure 2, Table S1).

Phylogenetic divergence between the two clusters was supported by the high separating SNPs number (average of 3307) between Cluster I and Cluster II strains (Table S1).

4. Discussion

TGC and ERV are two tetracyclines derivates potentially useful in the treatment of severe infections caused by VRE, especially of intra-abdominal infections [3,9].

In our contemporary collection of VRE clinical isolates we found a prevalence of TGC-R/ERV-R of 12.5% (4/32).

We used a phenotypic and genotypic approach (WGS) to investigate the features of TGC-R/ERV-R VRE isolates. All the TIG-R/ERV-R strains belonged to one of the major VREfm clones spread in clinical setting (ST80) [14–18] and we correlated the phenotype with the presence of an uncommon

mutation (the Y58D amino acid substitution) in the *rpsJ* gene encoding for the S10 ribosomal structural protein. This mutation has been previously associated with a reduced TGC susceptibility among *in vitro*-selected *E. faecium* mutants [7]. The association of TGR-R and ERV-R found in our collection support the hypothesis that the S10 ribosomal protein may be a general target of both TGC and ERV [4].

In our work, all TGC-R/ERV-R strains with the *rpsJ* mutation also carried *tet* genes. It has been reported that other mechanisms, such as the overexpression of *tet*(L) gene and *tet*(M) plasmid-encoded gene, can cause TGC non-susceptibility [19]. However, in our collection, strains carrying the *tet* genes but lacking the *rpsJ* mutation were TET-R and susceptible to TGC and ERV, thus supporting the primary role of *rpsJ* mutation.

Unfortunately, strains carrying the *rpsJ* mutation only (without *tet* genes) weren't isolated, therefore it was not possible to conclude whether *rpsJ* mutation could be sufficient to cause TGC-R and ERV-R. It is impossible to exclude a potential role of overexpression of *tet*(L) and *tet*(M) genes in the phenotype observed and a concomitant presence may be necessary [19,20] for a high-level resistance phenotype.

Phylogenetic analysis revealed considerable number of separating SNPs between the cluster of strains harbouring the mutation and those lacking it, indicating that they are segregated from each other. In fact, the two groups of strains formed distinct, divergent clusters in the phylogenetic tree.

Among possible limitations of the study, we should mention the relatively small number of isolates and the clonal nature of the collection. Finally, data on antibiotic therapy administered to patients were not available, therefore we were not able to speculate whether *rpsJ* mutanion evolved under selective pressure.

In summary, our data highlight the role of the substitution of a tyrosine (Y) with aspartic acid (D) in position 58 of S10 ribosomal protein as cause of TGC and ERV resistance in *E. faecium*.

The identification of such a substitution in the ribosomal S10 protein in clinical isolates of VREfm underscore the need for enhanced genomic surveillance in order to detect emerging resistance

mechanisms potentially involved in the resistance to last-resort agents, such as TGC and ERV. Also, the correlation of the Y58D mutation with observed resistance, especially when found alongside *tet*(L) and *tet*(M) genes, suggests that this mutation could serve as a resistance marker. Rapid detection of this mutation in VRE clinical isolates might guide clinicians in the selection of therapeutic regimens, potentially reducing the use of ineffective antibiotics while improving patient outcome.

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Conflict of interests: None declared.

Ethical approval: All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Author contributions: FV and FA conceived and designed the study. FV and EB performed the experiments and acquired data. FV, EB, GLB, MM and FA analysed and interpreted the data. FV drafted the article. FV, EB, GLB, MM and FA revised the article. All authors read and approved the final version of the manuscript.

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Supplementary materials

See Table S1.

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Table 1. Features of the 32 vancomycin-resistant Enterococci (VRE) included in the study.

Isolate	Isolation date	Source	Ward[a]	MLST[b]	MIC (mg/L)[c]				Detection of *tet* genes			RpsJ^{Y58D}
					TET[**]	TGC[*]	ERV[*]		*tet*(L)	*tet*(M)		
E. faecium VRE31	Sep 24	Urine sample	Hepatology	ST80	**>16**	**1**	**>0.5**		+	+		+
E. faecium VRE32	Dec 23	Blood culture	Hospital Medicine	ST80	≤0.5	0.5	0.12		–	–		–
E. faecium VRE33	Feb 24	Urine sample	ICU	ST117	≤0.5	0.25	0.06		–	–		–
E. faecium VRE35	Feb 24	Blood culture	Hepatology	ST117	≤0.5	0.5	0.06		–	–		–
E. faecium VRE36	Feb 24	Urine sample	ICU	ST117	≤0.5	0.25	0.12		–	–		–
E. faecium VRE37	Feb 24	Drainage liquid	Urology	ST80	≤0.5	0.5	0.06		–	–		–
E. faecium VRE38	Feb 24	Urine sample	ICU	ST117	≤0.5	0.5	0.06		–	–		–
E. faecium VRE39	Mar 24	Blood culture	ICU	ST1478	≤0.5	0.25	0.06		–	–		–
E. faecium VRE40	Mar 24	Urine sample	Infectious Diseases	ST80	**>16**	**1**	**>0.5**		+	+		+
E. faecium VRE41	Mar 24	Urine sample	Endocrinology	ST117	≤0.5	0.25	0.06		–	–		–
E. faecium VRE42	Mar 24	Urine sample	Hospital Medicine	ST117	≤0.5	0.25	0.06		–	–		–
E. faecium VRE43	Mar 24	Urine sample	University Medicine	ST80	≤0.5	0.25	0.12		–	–		–
E. faecium VRE44	Mar 24	Urine sample	Geriatrics	ST1478	≤0.5	0.5	0.25		–	–		–
E. faecalis VRE55	May 24	Blood culture	ICU	ST6	**>16**	0.25	0.25		–	+		–
E. faecium VRE56	May 24	Urine sample	ICU	ST117	≤0.5	0.25	0.06		–	–		–
E. faecium VRE57	May 24	Urine sample	Nephrology	ST1478	≤0.5	0.12	0.06		–	–		–
E. faecium VRE58	Jun 24	Urine sample	Gastroenterology	ST80	≤0.5	0.25	0.12		–	–		–
E. faecium VRE59	Jun 24	Urine sample	Emergency Department	ST80	≤0.5	0.25	0.12		–	–		–
E. faecalis VRE60	Jun 24	Urine sample	Hospital Medicine	ST6	**>16**	0.25	0.12		–	+		–
E. faecium VRE61	Jul 24	Blood culture	University Medicine	ST1478	≤0.5	0.25	0.25		–	–		–
E. faecium VRE62	Jun 24	Surgical sample	Hospital Medicine	ST1478	≤0.5	0.25	0.06		–	–		–
E. faecium VRE63	Jul 24	Surgical sample	General Medicine	ST80	**>16**	**1**	**>0.5**		+	+		+
E. faecium VRE74	Feb 24	Surgical sample	ICU	ST1478	≤0.5	0.12	0.06		–	–		–
E. faecium VRE75	Mar 24	Drainage liquid	General Medicine	ST80	≤0.5	0.25	0.12		–	–		–
E. faecium VRE96	Feb 24	Urine sample	General Medicine	ST80	≤0.5	0.12	0.06		–	–		–
E. faecium VRE97	Feb 24	Urine sample	ICU	ST1478	≤0.5	0.25	0.12		–	–		–
E. faecium VRE98	Feb 24	Urine sample	Urology	ST80	**>16**	0.12	0.12		+	+		–
E. faecium VRE99	Mar 24	Urine sample	Nephrology	ST80	≤0.5	0.06	0.12		–	–		–
E. faecium VRE100	Apr 24	Urine sample	Urology	ST80	**>16**	**1**	**>0.5**		+	+		+
E. faecium VRE101	Apr 24	Urine sample	Nephrology	ST80	≤0.5	0.25	0.12		–	–		–
E. faecium VRE102	Sep 24	Blood culture	Hospital Medicine	ST117	≤0.5	0.06	0.06		–	–		–
E. faecium VRE103	Sep 24	Blood culture	Hospital Medicine	ST80	**>16**	0.06	0.12		+	+		–

[a]ICU, intensive care unit.
[b]According to MLST Pasteur scheme.
[c]MIC, minimum inhibitory concentration; TET, tetracycline; TGC, tigecycline; ERV, eravacycline. Results were interpretated according to EUCAST (*) or CLSI (**) clinical breakpoints. MICs of TET, TGC and ERV are indicated in bold for resistant isolates.
+, presence of *tet* genes/*rpsJ* mutation; –, absence of *tet* genes/*rpsJ* mutation.

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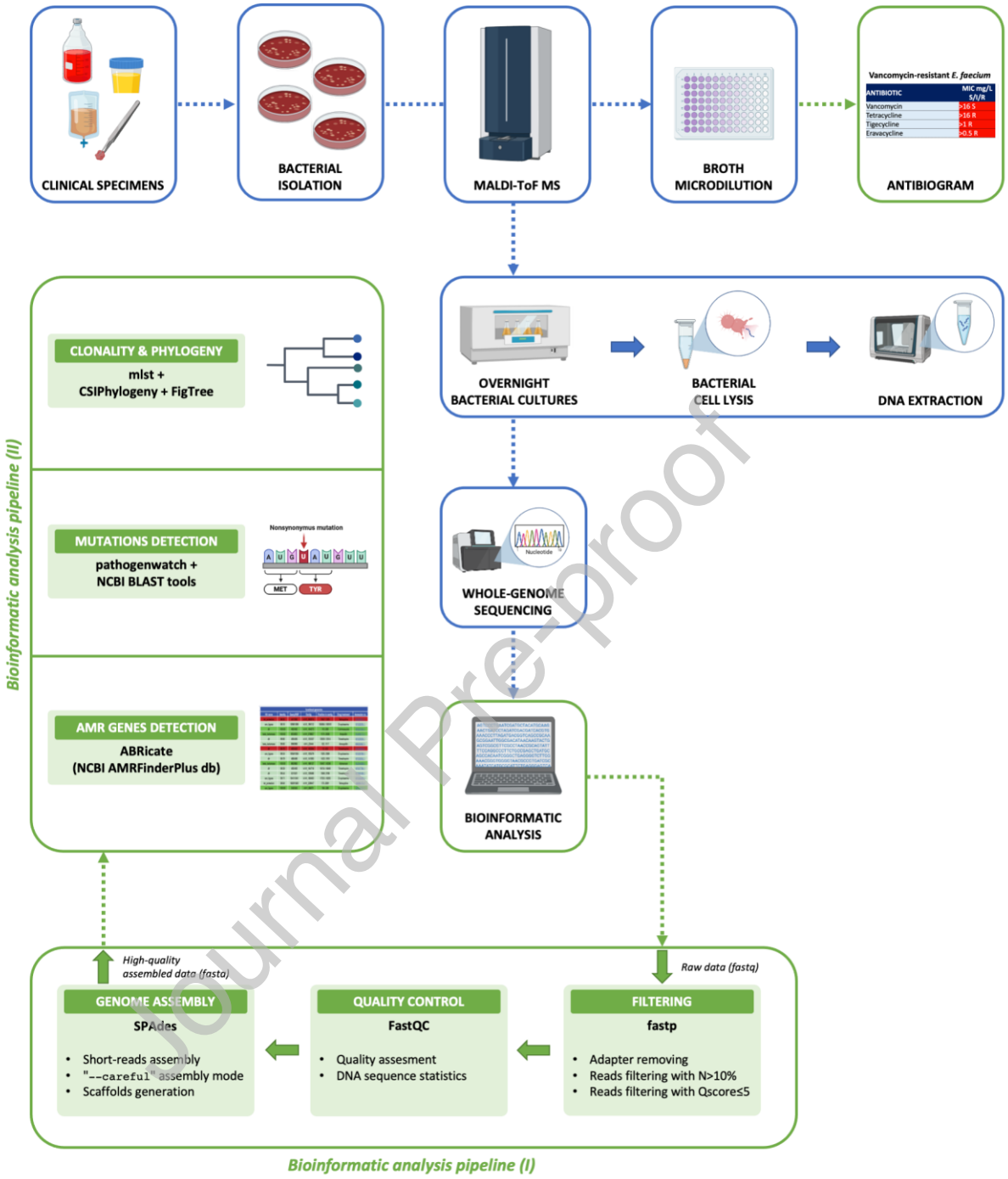

Figure 1. Overview of the experimental workflow adopted in the study. In blue wet-lab procedures, while in green dry-lab procedures.

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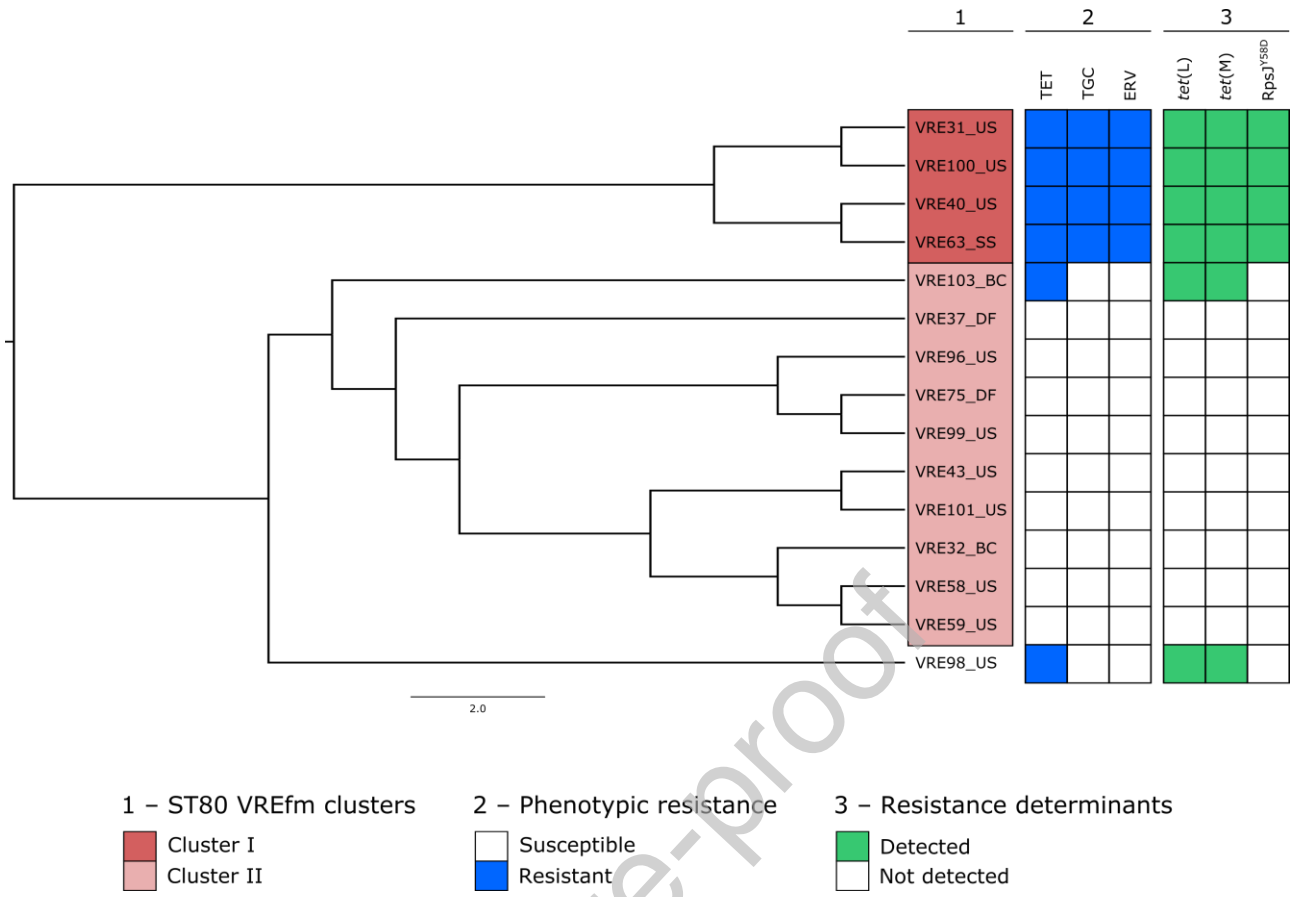

Figure 2. Midpoint-rooted phylogenetic tree of the 15 ST80 VREfm strains included in the study. The percentage of reference genome (VREfm ATCC 51559) covered by all isolates was 82.2%. A heatmap displays the two ST80 VREfm clusters, phenotypic resistance, and resistance determinants. US, urine sample; BC, blood culture; SS, surgical sample; DF, drainage fluid; TET, tetracycline; TGC, tigecycline; ERV, eravacycline.

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