PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVI and XXXVII cycles)

Name of PhD student: Cinthi Marzia

Title of PhD research: Isolation and molecular characterization of linezolid-resistant enterococci of human, animal, and environmental origin

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Sezione Microbiologia, DISVA

Cycle: XXXVII

PhD Curriculum: Biomolecular Sciences

ABSTRACT (1000 characters, including spaces):
Enterococci are members of gut microbiota of human and animals. Although regarded as commensals, they are cause of human opportunistic infections worldwide. The treatment of severe infections due to VRE and MDR enterococci is often limited to the last-resort antibiotics such as oxazolidinones: linezolid (LZD) and tedizolid (TDZ). Apart from ribosomal mutations, acquisition of resistance genes played a critical role in the spread of LZD resistance in isolates both of human and animal origin. These genes are often associated with mobile genetic elements (such as non-conjugative and conjugative plasmids, transposons, ICEs and prophages) which facilitate their dissemination.
Florfenicol, extensively used in livestock, could promote the spread of LZD resistance genes as a result of co-selection mechanisms with serious consequences for human health. This project aims to investigate possible reservoirs of LZD resistance genes both in animal and environmental setting.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

BACKGROUND

Enterococci are important nosocomial pathogens and Enterococci are members of gut microbial communities of animals, including humans. Although regarded as commensals, they are also a common cause of human opportunistic infections: specifically *Enterococcus faecalis* and *Enterococcus faecium* are the leading cause of healthcare-associated infections worldwide.
The success as nosocomial pathogens is due to their persistence in the environment and tolerance to disinfectants and to their intrinsic or acquired resistance to many drugs. The treatment of infections due to MDR- and vancomycin-resistant enterococci is limited to last resort antibiotics such as quinupristin/dalfopristin, oxazolidinones (linezolid and tedizolid), daptomycin which, however, are only approved for certain indications, and resistances to those drugs have already been reported.

Despite oxazolidinones have been approved only for clinical use an increasing number of linezolid-resistant enterococci has been detected in animal and environmental settings due to spread of acquired resistance genes [1-3, 5].
The data on sales of veterinary drugs indicate that in Italy the consumption of antibiotics is considerably higher than in other European states. The extensive use of florfenicol in livestock to prevent or treat bacterial infections can promote the spread of the resistance to phenicols, as well as to oxazolidinones, as a result of co-selection mechanisms, with serious consequences for human health.

SCIENTIFIC AIMS

The aim of project was to investigate the potential existence of reservoirs of linezolid resistance genes both in animal and environmental setting. The presence of animal reservoirs of oxazolidinone resistance genes, potentially transmissible to human pathogens, is cause for concern because of the risk of spread of antibiotic-resistant isolates to humans via the food chain. The currently worrisome situation in humans may be the tip of the iceberg of a more widespread phenomenon. This research helps to clarify the role of animal and environmental enterococci as a source of LZD resistance genes transferable to major human pathogens. This research, that provides data on the dynamics of onset and spread of LZD resistance in non-clinical enterococci, is crucial to explain the role of certain reservoirs in whose absence resistances would probably fail to emerge in human pathogens.

WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

To investigate the occurrence, the genetic environments and the transferability of oxazolidinone resistance genes in enterococci of environmental origin.

Methods.

Sampling, sample processing and enterococcal isolation.
Sampling activities were carried out in February 2021 at an area 2 km from the Salinello estuary, a river in Abruzzo region flowing into the Adriatic Sea. Near to this river stretch there are an urban area and a wastewater treatment plan.
Overall, 10 freshwater samples of 500 ml each have been collected over the month. The specimens have been treated as previously described [Fioriti S, *et al*. 2021].
PCR assays and susceptibility tests. The strains were screened by PCR for the presence of *cfr/cfr*-like, *optrA* and *poxtA* genes [5]. The amplicons were subjected to Sanger sequencing. Susceptibility to several antibiotics was performed by standard broth microdilution assay and Etest.
S1-PFGE, southern blotting, and hybridization assays. S1-PFGE, Southern blotting and hybridization with a specific probes have been used to assess the plasmidome and determine the gene location.
Detection of circular forms. To investigate the excision of the genetic contexts, inverse PCR assay were performed using outward-directed primer pair targeting the linezolid resistance genes [4].
WGS and sequence analysis. The genomic DNA was extracted by the QIAcube automated extractor (Qiagen). Extracted DNA was subjected to WGS.
Mating experiments. Conjugative transfer of linezolid resistance genes was assessed both by filter mating experiment and in aquaria microcosm assays using *E. faecium* 64/3 and *E. faecium* Ef1 as recipients.

Expected/Obtained Results.

PCR experiments Just one florfenicol-resistant enterococcus, *E. faecium* M1, was found positive for the presence of *poxtA* gene. The isolate, identified by MALDI-TOF (Vitek-MS, bioMérieux), carried only the *poxtA* gene and Sanger sequencing showed that was identical to the relevant wild-type sequence.
Antimicrobial susceptibility profile. *E. faecium* M1 was resistant to florfenicol (64 mg/L), chloramphenicol (32 mg/L), erythromycin (>128 mg/L) and tetracycline (64 mg/L). However, the isolate was intermediate to linezolid (MIC, 4 mg/L) and susceptible to tedizolid (0.5 mg/L) and vancomycin (1 mg/L).
Location of *poxtA* gene and detection of circular forms. Hybridisation occurred both on chromosome and on five plasmids (from 4.3 to 194 kb). Inverse PCR experiments indicated that a circular form of *poxtA*

genetic context was detectable suggesting the instability of this genetic environment and an intracellular mobility of the *poxtA* gene.

WGS analysis. Bioinformatic analysis indicated that the *poxtA* gene was on a 30,877-bp plasmid (named pEfM1) with high identity (nucleotide identity 99%; cover 100%) and synteny to the to the enterococcal 27-kb plasmid pEfm-Ef3 detected in an *E. faecium* isolate from sediment collected in an Italian coastal area [4]. In both plasmids the *poxtA* genetic context, flanked by two IS1216, was located in a Tn*6657*-like transposon also containing *fexB* gene, *tet*(L) and *tet*(M) genes, in tandem, were located upstream of the Tn*6657*-like. The comparative analysis of the two plasmids showed that in pEfM1 a second copy of *poxtA* genetic context was inserted within the Tn*6657*-like upstream of the *orf8*, maybe thanks to IS1216-mediated recombination phenomena (Figure 1). The strain belonged to ST1036 which has been associated with human enterococci (PubMLST).

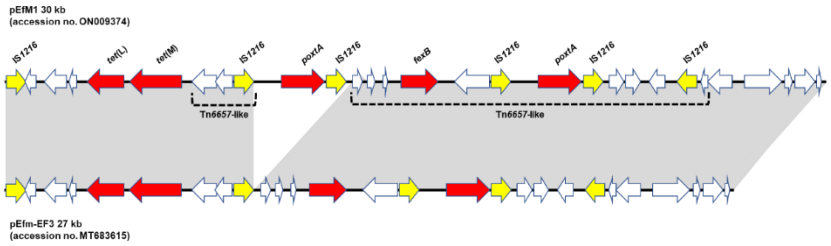

Figure 1. Schematic representation and comparison between the *E. faecium* pEfm1 plasmid and the *E. faecium* pEfm-EF3 plasmid.

Transferability in filter mating experiments and in aquaria microcosm assays. In filter mating experiments, *E. faecium* M1 was able to transfer the *poxtA* gene to *E. faecium* 64/3 and *E. faecium* Ef1 recipients with frequencies of $5.8x10^{-2}$ and $5x10^{-3}$, respectively. In aquaria microcosm assays, *poxtA* gene was successfully transferred only to *E. faecium* 64/3 recipient with frequencies ranged from $5.0x10^{-4}$ to $2.9x10^{-7}$. For each transfer experiment, three transconjugants were randomly selected and characterized for the presence of *poxtA* gene and for their antibiotic susceptibility (Table 1).

		Recipient	Transconjugant	Transfer Frequency	*poxtA*	MIC (mg/L) of:[e]						
						FFC	CHL	LZD	TZD	TE	ERY	VAN
Filter mating experiments												
		E. faecium 64/3	T-1	$5.8x10^{-2}$	+	64	32	4	0.5	128	128	0.5
		E. faecium Ef1	T-5	$5x10^{-3}$	+	128	64	8	1	128	>128	1
Aquaria microcosm experiments												
Aquarium A[a]	24 hours	*E. faecium* 64/3	TC-2	$6.3x10^{-5}$	+	64	32	4	0.5	128	>128	0.5
	48 hours	*E. faecium* 64/3	TC-3	$1.8x10^{-7}$	+	64	32	4	0.5	128	>128	0.5
	96 hours	*E. faecium* 64/3	-	ND[d]								
Aquarium B[b]	24 hours	*E. faecium* 64/3	TC-6	$5x10^{-4}$	+	64	32	4	0.5	128	>128	0.5
	48 hours	*E. faecium* 64/3	TC-7	$2.7x10^{-7}$	+	64	32	4	0.5	128	>128	0.5
	96 hours	*E. faecium* 64/3	-	ND								
Aquarium C[c]	24 hours	*E. faecium* 64/3	TC-8	$9.2x10^{-4}$	+	64	32	4	0.5	128	>128	0.5
	48 hours	*E. faecium* 64/3	TC-9	$2.9x10^{-7}$	+	64	32	4	0.5	128	>128	0.5
	96 hours	*E. faecium* 64/3	-	ND								
Aquarium A	24 hours	*E. faecium* Ef1	-	ND								
	48 hours	*E. faecium* Ef1	-	ND								
	96 hours	*E. faecium* Ef1	-	ND								
Aquarium B	24 hours	*E. faecium* Ef1	-	ND								
	48 hours	*E. faecium* Ef1	-	ND								
	96 hours	*E. faecium* Ef1	-	ND								
Aquarium C	24 hours	*E. faecium* Ef1	-	ND								
	48 hours	*E. faecium* Ef1	-	ND								
	96 hours	*E. faecium* Ef1	-	ND								

Table 1. Antimicrobial susceptibility profiles, frequencies of conjugation for *E. faecium* M1 and its transconjugants.
[a]containing freshwater; [b]containing osmotic water; [c]containing osmotic water with florfenicol (0.05 mg/L); [d]not detectable.
[e]FFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TZD, tedizolid; TE, tetracycline; ERY, erythromycin; VAN, vancomycin.

WP 2. Objective.
To investigate the occurrence, the genetic environments and the transferability of oxazolidinone resistance genes in enterococci of animal and human origin.

Methods.
Bacterial Strains. Florfenicol-resistant enterococci recovered both from wild animals (boars and raptors) and from livestock, where florfenicol was regularly used, were sampled in collaboration with the "Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche". In parallel, linezolid-resistant clinical enterococci from the Infectious Diseases Clinic (Marche Polytechnic University) were collected.
PCR assays and susceptibility tests. The strains was screened by PCR and Sanger assays for the presence of *cfr*/*cfr*-like, *optrA* and *poxtA* genes [5]. Susceptibility to several antibiotics was performed by standard broth microdilution assay.

Expected/Obtained Results.
A total of 18 enterococci carrying LZD resistance genes have been collected and characterized for their susceptibility to date. Only 7/18 isolates have undergone WGS analysis and transferability assays. The results are summarized in Table 2.

Strain	Species/ST	Source	Gene/localization				MIC (mg/L) of: [c]				Transfer Frequency	Genetic element/contest
			poxtA	*optrA*	*cfr*	*cfrD*	FFC	CHL	LZD	TDZ		
V1225	*E. faecalis*	chicken	-	+	-	-	128	64	2	0.5	-	-
V1339	*E. faecium*	boar	-	+	-	-	64	64	4	1	-	-
V1343	*E. durans*	boar	+	-	-	-	128	32	4	1	-	-
V1344	*E. faecalis*	boar	-	+	-	-	128	64	4	1	-	-
V1507	*E. faecalis*	bovine	-	+	-	-	128	128	4	1	-	-
V1460	*E. thailandicus*	swine	+	+	-	-	64	64	2	1	-	-
V1461	*E. faecalis*	swine	-	+	-	-	64	64	4	1	-	-
V1462	*E. avium*	swine	-	+	+	-	16	16	2	0.5	-	-
V1463	*E. faecium*	swine	-	+	-	-	32	32	2	0.5	-	-
714114	*E. faecalis*	patient	-	+	-	-	64	64	16	4	-	-
745599	*E. faecalis*	patient	-	+	-	-	64	128	8	2	-	-
30488	*E.faecalis*/ST476	raptor	-	+/c [a]	-	-	64	64	8	0.5	ND [d]	Tn*6674*-like
FS4	*E. gallinarum*	swine	+/38kb [b]	+/34kb	+/34kb	-	128	32	8	4	ND	pEgFS4-1/pEgFS4-2
R1	*E. hirae*	swine	+/17kb	-	-	-	64	32	4	1	ND	pEh-R1
V378	*E. casseliflavus*	swine	+/80kb	-	-	+/80kb	32	32	8	2	ND	pEc378
V386	*E. faecalis*/ST32	swine	+/34kb	-	-	+/34kb	128	128	2	2	$2.3x10^{-1}$	pV386
V392	*E. faecalis*	swine	+/34kb	-	-	+/34kb	64	128	4	2	$8.0x10^{-2}$	pV386
V308	*E. casseliflavus*	swine	+/34kb	-	-	+/34kb	128	64	4	1	$2.3x10^{-2}$	pV386

Table 2. Typing data, LZD resistance genes and their location, antimicrobial susceptibility profiles, and transferability of 18 enterococci of human and animal origin.
[a] c, chromosome;
[b] plasmid size (in kb);
[c] FFC, florfenicol; CHL, chloramphenicol; LZD, linezolid; TZD, tedizolid
[d] ND, not detectable transfer under conditions used;

The genome and the transferability of linezolid resistance of other isolates will be analyzed shortly. Furthermore, new samplings will be performed both in clinical and animal setting.

REFERENCES

1. Brenciani A, *et al*. 2022. Oxazolidinones: mechanisms of resistance and mobile genetic elements involved. J Antimicrob Chemother. In press.
2. Fioriti S, *et al*. Detection of oxazolidinone resistance genes and characterization of genetic environments in enterococci of swine origin, Italy. 2021. *Microorganisms* doi.10.3390/microorganisms8122021.
3. Biggel, M, *et al*. (2021) Genetic context of *optrA* and *poxtA* in florfenicol-resistant enterococci isolated from flowing surface water in Switzerland. *Antimicrob Agents Chemother* 65, e0108321.
4. Morroni G, *et al*. Characterization of a Multiresistance Plasmid Carrying the *optrA* and *cfr* Resistance Genes from an *Enterococcus faecium* Clinical Isolate. 2018. *Front Microbiol*. DOI: 10.3389/fmicb.2018.02189.

5. Fioriti S, *et al.* 2021. Linezolid resistance genes in enterococci isolated from sediment and zooplankton in two Italian coastal areas. *Appl Environ Microbiol*. 87: e02958-20.
6. Cinthi M, *et al.* 2022. Occurrence of a plasmid cocarrying *cfr(D)* and *poxtA2* linezolid resistance genes in *Enterococcus faecalis* and *Enterococcus casseliflavus* from porcine manure, Italy. *J Antimicrob Chemother*. 77: 598–603.

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Course: Design of research: European projects (Prof. Paone – gennaio 2022).

2. Seminar: La rivoluzione deliberata in crioelettrone-microscopia, biologia strutturale e scienze della vita (Prof. Bolognesi- 07/06/22 PhD week 2022).

3. Seminar: Current threats to research ethics and how to cope with them (Prof. Seeber- 09/06/22 PhD week 2022).

4. Individual internship: Characterization of oxazolidinone-resistant clinical bacteria: from phenotype to Whole Genome Sequencing (Prof. Morroni – 15 giugno-15 luglio 2022).

List of periods spent abroad

1.

2.

...

List of conferences/workshops attended and of contributions eventually presented

1. Conference and poster presentation: 50° CONGRESSO NAZIONALE SIM 2022 (18 - 21 Settembre 2022).

2.

...

Part 3. PhD student information on publications

If not yet published, please indicate the publication status (submitted, accepted, in preparation...)

List of publications on international journals

J1. Coccitto, S.N., Cinthi, M., Fioriti, S., Morroni, G., Simoni, S., Vignaroli, C., Garofalo, C., Mingoia, M., Brenciani, A., Giovanetti, E. "Linezolid-resistant *Enterococcus gallinarum* isolate of swine origin carrying *cfr*, *optrA* and *poxtA* genes" J Antimicrob Chemother, 77, 331-337 (2022) DOI: 10.1093/jac/dkab408.

J2. Cinthi, M., Coccitto, S.N., D'Achille, G., Morroni, G., Simoni, S., Fioriti, S., Magistrali, C.F., Brenciani, A., Giovanetti, E. "Characterization of a novel *cfr(D)/poxtA*-carrying plasmid in an oxazolidinone-resistant *Enterococcus casseliflavus* isolate from swine manure, Italy" J Glob Antimicrob Resist, 30, 308-310 (2022) DOI: 10.1016/j.jgar.2022.07.007.

J3. Cinthi, M., Coccitto, S.N., Fioriti, S., Morroni, G., Simoni, S., Vignaroli, C., Magistrali, C.F., Albini, E., Brenciani, A., Giovanetti, E. "Occurrence of a plasmid co-carrying *cfr*(D) and *poxtA2* linezolid resistance genes in *Enterococcus faecalis* and *Enterococcus casseliflavus* from porcine manure, Italy" J Antimicrob Chemother, 77:598-603 (2022) DOI: 10.1093/jac/dkab456.

J4. Cinthi, M., Coccitto, S.N., Morroni, G., D'Achille, G., Brenciani, A., Giovanetti, E. "Detection of an *Enterococcus faecium* carrying a double copy of the *poxtA* gene from freshwater river, Italy" publication status: in preparation.

J5. Albini, E., Coccitto, S.N., Cinthi, M., Giovannetti, E., Gobbi, M., Massacci, F.R., Pavone S., Brenciani, A. "*optrA* -mediated linezolid resistance in an *Enterococcus faecalis* isolate recovered from a wild raptor (*Falco peregrinus peregrinus*), central Italy" Journal of Global Antimicrobial Resistance. Publication status: submitted.

J6. Cinthi, M., Coccitto, S.N., Simoni, S., Garofalo, C., Vignaroli, C., Brenciani, A., Giovanetti, E. "Detection of phenicol-oxazolidinone resistance gene *poxtA* in *Enterococcus hirae* from swine faeces, Italy" M Drug Resistance. Publication status: submitted.

J7. Coccitto, S.N., Cinthi, M., Morroni, G., Pocognoli, A., Simoni, S., D'Achille, G., Giovanetti E. "Co-existence of *cfr* and *fosB* genes in a MDR *Staphylococcus hominis* blood isolate from an Italian hospital" Journal of Global Antimicrobial Resistance. Publication status: accepted.

List of publications on conference proceedings

List of other publications (books, book chapters, patents)

12-10-2022

Student signature

Supervisor signature