PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

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

Name of PhD student: Marzia Cinthi

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

Name of PhD supervisor: Eleonora Giovanetti

Research lab name: -Sezione Microbiologia, Dipartimento di Scienze Biomediche e Sanità Pubblica
-Sezione Microbiologia, DISVA

Cycle: XXXVII

PhD Curriculum: Biomolecular Sciences

ABSTRACT (1000 characters, including spaces):
Oxazolidinones, linezolid and tedizolid, are last-resort antibiotics used to treat severe human infections due to MDR Gram-positive bacteria including vancomycin-resistant enterococci. These antimicrobials inhibit the protein synthesis by binding to the peptidyl transferase centre of the 50S ribosomal subunit. Oxazolidinone resistance can arise through the acquisition of several transferable resistance genes conferring decreased susceptibility to linezolid and phenicols. Florfenicol, approved only for veterinary use and routinely administered to livestock, could promote the spread of linezolid resistance in animal setting as a result of co-selection mechanisms. Due to their ubiquity in animal faeces and persistence in the environment, enterococci spread in many habitats and can be isolated from the soil, water, food of animal origin with serious consequences for human health. The purpose of this study was to investigate the occurrence of linezolid resistance genes in enterococci of animal origin.

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

BACKGROUND

Enterococci are commonly found in the gastrointestinal tract of healthy humans and animals, but they also are among the most frequent causes of hospital-acquired infections, particularly *Enterococcus faecalis* and *Enterococcus faecium*. Enterococcal infections include those of the urinary tract, bloodstream, endocardium, and wounds. Antibiotic resistance among enterococci is increasing and considerably limiting the available therapeutic options: oxazolidinones represent one of the few remaining treatment options for infections caused by VRE and MDR enterococci [1]. Besides the occurrence of linezolid-resistant enterococci in hospitals, their detection in other reservoirs is of special concern since the potential transfer of oxazolidinone resistance genes from enteric bacteria in animals to humans via the food chain could represent a serious public health problem. Furthermore, manure-associated enterococci can be disseminated into water resources, representing an important source of contamination.

SCIENTIFIC AIMS

The occurrence of animal reservoirs of oxazolidinone resistance genes, potentially transmissible to human pathogens via food chain, is cause for concern. The aim of the project was to investigate the presence of oxazolidinones resistance genes and their genetic contexts in enterococci of bovine and swine origin in order to gain insight into their dissemination dynamics. This research also contributed to clarifying the role of animal enterococci as a source of linezolid resistance genes to major human pathogens via mobile genetic elements [1].

WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

To investigate the occurrence of oxazolidinone resistance genes in enterococci of bovine origin, to describe their genetic environments and to analyse their transferability.

Methods.

Sampling procedures and strains isolation. During surveillance 66 pooled faecal samples were collected from cattle farms where florfenicol (FFC) was previously used. Each pooled faecal sample was inoculated in 5 mL of buffered peptone water supplemented with FFC (10 mg/L) and incubated at 44°C for 48 h. Then, 100 µL were inoculated on Slanetz-Bartley agar plates supplemented with FFC (10 mg/L) for the selection of resistant enterococci.

Genotypic and phenotypic characterization. FFC-resistant enterococci were screened by PCR for the presence of *cfr* and its variants, *optrA* and *poxtA* genes [2]. Isolates were tested for their susceptibility to FFC, chloramphenicol, linezolid, tedizolid, tetracycline, erythromycin, and vancomycin by standard broth microdilution assay. Identification of enterococci was carried out by Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF).

Detection of circular forms. To investigate the excision of the linezolid resistance genes contexts, PCR mapping assays were performed using outward-directed primer pairs targeting the *optrA* (5'- TTTTTCCACATCCATTTCTACC-3' and 5' - GAAAAATAACACAGTAAAAGGC - 3') and *poxtA* (5'- GACGAGCCGACCAACCACCT-3' and 5'- TTGGATTTTTGTCCGCCTGAA - 3') genes.

Mating experiments. Conjugal transfer was performed on a membrane filter as described previously [2].

WGS analysis. The genomic DNA was extracted by the QIAcube automated extractor (Qiagen). Extracted DNA was subjected to WGS.

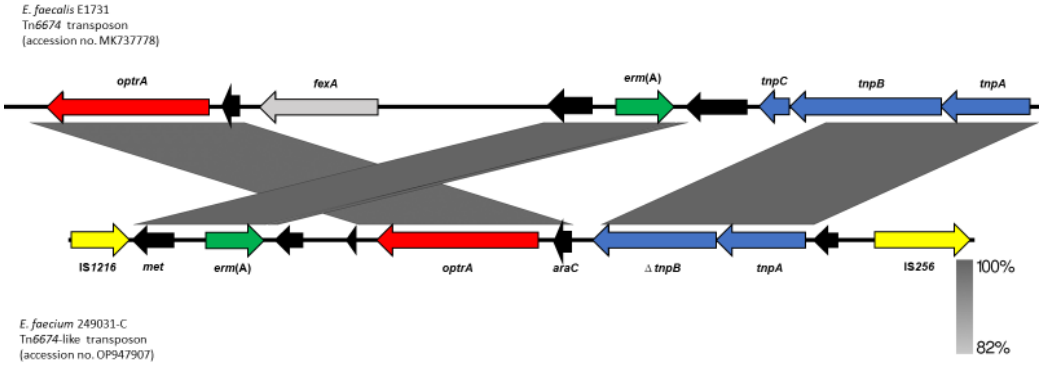
Expected/Obtained Results.

Linezolid resistance determinants and antibiotic susceptibility. Overall, 18 FFC-resistant enterococci were isolated from 66 faecal samples. PCR screening showed that only *E. faecium* 249031-C was positive for the presence of the *poxtA* and *optrA* genes. The isolate exhibited resistance to FFC (MIC, 128 mg/L), chloramphenicol (MIC, 32 mg/L), tedizolid (MIC, 2 mg/L), erythromycin (MIC, >128 mg/L), and tetracycline (MIC, 128 mg/L) . However, it was intermediate to linezolid (MIC, 4 mg/L) and susceptible to vancomycin (MIC, 1 mg/L).

Transferability of the *poxtA* and *optrA* genes. The strain was able to co-transfer the *optrA* and *poxtA* genes to *E. faecium* 64-3 recipient with high frequency (2.9×10^{-3} per recipient). Two transconjugants randomly selected were characterized for the presence of *optrA* and *poxtA* by PCR. They exhibited resistance to FFC, chloramphenicol, erythromycin and tetracycline, and susceptibility or reduced susceptibility to linezolid, and tedizolid.

Genome bioinformatic analysis. *E. faecium* 249031-C (ST22) harboured two plasmids: the *optrA*-carrying p249031-S (179,049 bp) and the *poxtA*-carrying p1818-c (23,864 bp). p249031-S showed a high degree of identity (98%) and coverage (100%) to the plasmid pF88_1 of *E. faecium* F88 isolated from river water in Switzerland [3]. Unlike the

pF88_1, p249031-S was characterized by the lack of a 67-kb resistance region containing *erm*(B), *ant(6)-Ia*, *ant(9)*, *aac(6')-aph(2'') and aph(3')*, *lsa*(E), *lnu*(B) and *sat4* genes. In p249031-S, the *optrA* gene was in a 11,319-bp region, flanking by IS*1216* and IS*256* with the same polarity, that displayed 100% DNA identity (coverage, 55%) to the corresponding one of the *optrA*-carrying Tn*6674* transposon first described in the chromosome of the porcine *E. faecalis* E1731 [4]. In *E. faecium* 249031-C, the Tn*6674*-like transposon consisted of a truncated *tnpB*, *optrA*, *erm*(A) and *met* genes; *fexA*, *spc* and *tpnC* were missing (Figure 1).



Inverse PCR experiments, using outward-directed primer pairs targeting the *optrA* gene, showed that a circular form of this region was detectable. Two amino acid changes (Y176D and G393D) were identified in the OptrA protein compared to the WT sequence $OptrA_{E349}$; this variant, called DD, was previously described [5]. The plasmid, belonging to the rep1 and repUS15 replicon types, showed a complete conjugation region. p1818-c was previously detected in *E. faecium* 1818 from a healthy human in Switzerland [6]. The *poxtA* and *fexB* genes are co-located on the Tn*6657* transposon, bounded by two copies of IS*1216* in the same orientation, in turn inserted in the Tn*6349* first described in the human *S. aureus* AOUC-0915 [7]. Inverse PCR experiments showed that a circular form was detectable. Since a complete conjugation region was detected in p249031-S, but not in p1818-c, it could possibly mobilize the second non-conjugative plasmid.

WP 2. Objective.

To characterize two plasmids co-carrying *cfr*(D)/*optrA* and *cfr*(D2)/*poxtA* linezolid resistance genes from two *Enterococcus avium* strains isolated from swine brain.

Methods.

Bacterial strains. Two *E. avium* isolates from two cases of sudden death in piglets (38157 and 44917). The piglets belonged to two unrelated farms in Umbria and were sent to IZSUM for diagnostic purposes. The samples were plated on blood agar plates and incubated at 37 ◦C for 2 days. Colonies were identified by MALDI-TOF. Both strains were isolated from swine brain.

Genotypic and phenotypic characterization. The strains were screened by PCR for the presence of known transferable oxazolidinone resistance genes [2]. Susceptibility tests were performed for tedizolid, FFC, chloramphenicol, linezolid, tetracycline, erythromycin and vancomycin by standard broth microdilution.

Detection of circular forms. To investigate the excision of the linezolid resistance genes contexts,
PCR mapping assays were performed using outward-directed primer pairs targeting the linezolid resistance genes: *cfr*(D) (5'-TTCCTAAAATAAAACGACTA- 3'and 5'-TACAAAAAGATTCCCAGCCA-3'), *optrA* (5'-GAAAAATAACACAGTAAAAGGC-3'and 5'-TTTTTCCACATCCATTTCTACC-3'), and *poxtA* (5'-GACGAGCCGACCAACCACCT -3' and 5'- TTCAGGCGGACAAAAATCCAA-3').

WGS analysis. The genomic DNA was extracted by the QIAcube automated extractor (Qiagen). Extracted DNA was subjected to WGS.

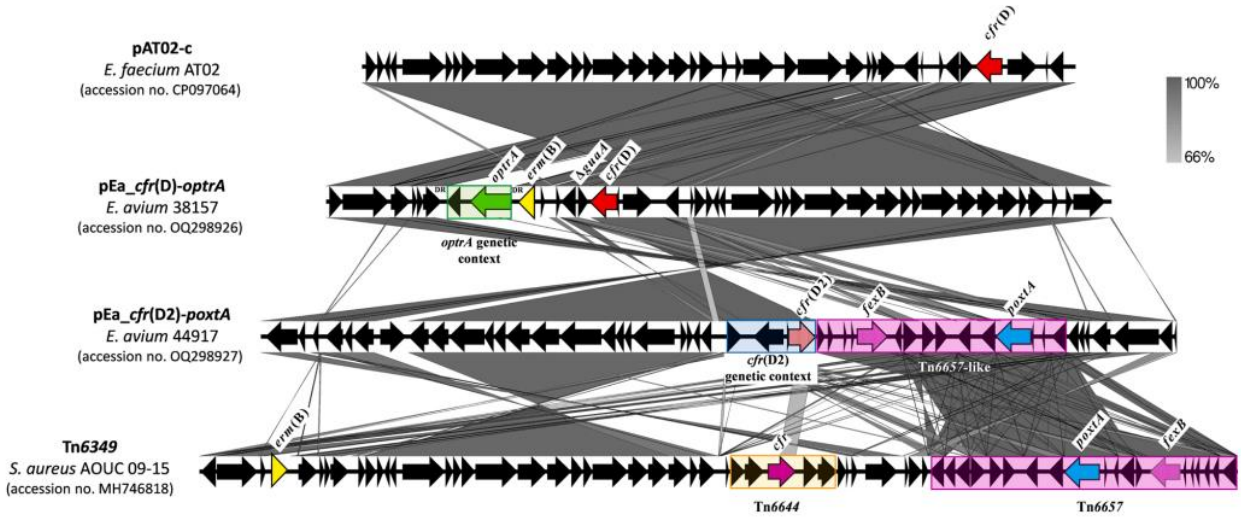
Mating experiments. Conjugal transfer was performed on a membrane filter as described previously [2].

Expected/Obtained Results.

Detection of oxazolidinone resistance genes and antimicrobial susceptibility testing. *E. avium* 38157 was found positive for the presence of *cfr*(D) and *optrA* genes. The isolate was resistant to erythromycin (MIC, >128 mg/L) and tetracycline (MIC, >128 mg/L), had reduced susceptibility to linezolid (MIC, 4 mg/L) and was susceptible to tedizolid (MIC, 0.5 mg/L), FFC (MIC, 8 mg/L), chloramphenicol (MIC, 16 mg/L), and vancomycin (MIC, 2 mg/L). PCR screening showed that *E. avium* 44917 was *cfr*(D) and *poxtA* positive. The isolate was resistant to FFC (MIC, 64

mg/L), chloramphenicol (MIC, 64 mg/L), tetracycline (MIC, 64 mg/L) and erythromycin (MIC, >128 mg/L) and susceptible to linezolid (MIC, 2 mg/L), tedizolid (MIC, 0.25 mg/L) and vancomycin (MIC, 0.5 mg/L).

WGS and bioinformatic analysis. In *E. avium* 38157 WGS analysis indicated that the *cfr*(D) and *optrA* genes were collocated on a 36,573-bp plasmid (34% GC content) designated pEa_*cfr*(D)-*optrA*. This plasmid, belonging to the Rep1 replicon type, showed high nucleotide identity with the 33.2-kb pAT02-c plasmid (accession no. CP097064) of *E. faecium* AT02 isolate from pet food. Since the pAT02-c plasmid only carries the *cfr*(D) gene, it can be assumed that the *optrA* genetic context integration, resulting in the pEa_*cfr*(D)-*optrA* plasmid, occurred later on. Nevertheless, inverse PCR experiments, using outward-directed primer pairs targeting the *optrA* gene, showed that its genetic context was unable to excise in circular form suggesting a stable acquisition. *E. avium* 38157 exhibited an *optrA* variant previously described in enterococci from human and pig origin in Italy [8]. The *cfr*(D) genetic context was flanking by two *IS*1216 elements with opposite orientation (Figure 2). No circular intermediate was detected.



The *E. avium* 44917 harboured a *cfr*(D)- and *poxtA*-carrying plasmid (42,657 bp) designated pEa_*cfr*(D2)-*poxtA*. This plasmid was 97% identical (coverage 85%) to the MDR composite transposon Tn*6349* (48 kb) also including the *poxtA*- and *fexB*-carrying Tn*6657*, first described in the human MRSA AOUC-0915 [7]. However, in pEa_*cfr*(D2)-*poxtA*, the Tn*6657* was 2.7 kb shorter and the *poxtA* and *fexB* genes, showing opposite orientation (Figure 2). The *poxtA* genetic context, flanked by IS*1216* with the same polarity, was highly conserved; inverse PCR experiments and sequencing showed that a circular form was detectable. Interestingly, bioinformatic analysis revealed the presence of a *cfr*(D)-like gene, named *cfr*(D2), which was shorter than *cfr*(D) wildtype due to the loss of 21 bp to the 3'- end. Therefore, Cfr(D2) (352 amino acids) differed from wildtype (357 amino acids) by the presence of a histidine (H) that replaced the last six amino acids (TIQVND). No circular intermediate was detected. Moreover, the *cfr*(D2) context, inserted upstream of the Tn*6657*-like transposon, fully replaced the Tn*6644* transposon that in *S. aureus* AOUC-0915 harbor the *cfr* gene flanking by two IS*Enfa5* transposases [7]. The pEa_*cfr*(D2)-*poxtA* plasmid, belonging to the Rep1 replicon type, had a complete transfer machinery responsible of the conjugation process.

Transfer experiments. Despite several attempts, *E. avium* 38157 was unable to transfer then pEa_*cfr*(D)-*optrA* plasmid to *E. faecalis* JH2-2 and *E. faecium* 64/3 recipients. Conversely, *E. avium* 44917 was able to move the pEa_*cfr*(D2)-*poxtA* plasmid to both *E. faecalis* JH2-2 and *E. faecium* 64/3 recipients at frequencies of 8.2×10^{-4} and 4.5×10^{-6}, respectively. For each mating experiment, two randomly selected transconjugants were analysed for their genotype, phenotype and genetic background. Transconjugants exhibited resistance to FFC, chloramphenicol and tedizolid and susceptibility to linezolid, erythromycin and tetracycline. PCR and Sanger sequencing indicated that all transconjugants acquired both *cfr*(D2) and *poxtA* genes.

REFERENCES

1. Brenciani A, *et al*. 2022. Oxazolidinones: mechanisms of resistance and mobile genetic elements involved. J Antimicrob Chemother https://doi.org/10.1093/jac/dkac263
2. Brenciani A., *et al*. 2019. Detection in Italy of a porcine *Enterococcus faecium* isolate carrying the novel phenicol-oxazolidinone-tetracycline resistance gene *poxtA*. J. Antimicrob. Chemother., 74, 817–818
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. Li *et al*. 2019. Tn*6674* is a novel enterococcal *optrA*-carrying multiresistance transposon of the Tn*554* family. Antimicrob. Agents Chemother., 63, e00809-19

5. Morroni G., *et al*. 2017. Commentary: nationwide surveillance of novel oxazolidinone resistance gene *optrA* in Enterococcus isolates in China from 2004 to 2014. Front. Microbiol., 8, 1631
6. Nüesch-Inderbinen M., *et al*. 2022. Faecal carriage of enterococci harbouring oxazolidinone resistance genes among healthy humans in the community in Switzerland. J Antimicrob. Chemother., 77, 2779–2783
7. D'Andrea, *et al*.2019.Characterization of Tn*6349*, a novel mosaic transposon carrying *poxtA*, and other resistance determinants, inserted in the chromosome of an ST5-MRSA-II strain of clinical origin. J. Antimicrob. Chem.,74, 2870–2875
8. Fioriti, S., *et al*. 2020. Detection of oxazolidinone resistance genes and characterization of genetic environments in enterococci of swine origin, Italy. Microorganims 8, 2021

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: Introduzione all'ambiente Latex per la redazione di documenti scientifici (Prof. Spinozzi, ottobre 2022).

2. Course: Theory and application of complex networks (Prof. Ortore, ottobre-novembre 2022)

3. Course: Alimentazione e salute: studio della qualità nutrizionale degli alimenti (Prof. Bacchetti, ottobre-dicembre 2022)

4. Molecular Methods in identification, quantification and Typing of bacterial isolates (Prof. Vignaroli, giugno 2023)

5. Course: Structural and functional aspects or RNA and RNA-proteins interactions (Prof. La Teana, luglio-settembre 2023)

List of periods spent abroad

1.

2.

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List of conferences/workshops attended and of contributions eventually presented

1. Conference: 51° CONGRESSO NAZIONALE della Società Italiana di Microbiologia (SIM) (24 - 27 Settembre 2023).

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., Massacci, F.R., Cinthi, M., Albini, E., Cucco, L., D'Achille, G., Morroni, G., Mingoia, M., Orsini, M., Giovanetti, E., Brenciani A., Magistrali C.F. "Co-location of the oxazolidinone resistance *poxtA*2 and *cfr*(D) genes on a multiresistance plasmid from a porcine *Streptococcus dysgalactiae* subsp. *equisimilis*, Italy" J Antimicrob Chemother, 2023;78:2099-2102. doi: 10.1093/jac/dkad169.

J2. Brescini, L., Fioriti, S., Coccitto, S.N., Cinthi, M., Mingoia, M., Cirioni, O., Giacometti, A., Giovanetti, E., Morroni, G., Brenciani A. "Genomic Analysis of a Linezolid-Resistant *Staphylococcus capitis* Causing Bacteremia: Report from a University Hospital in Central Italy" Microb Drug Resist, 2023;29:388-391. doi: 10.1089/mdr.2022.0330. Epub 2023 May 24.

J3. Cinthi, M., Coccitto, S.N., Simoni, S., Vignaroli, C., Brenciani, A., Giovanetti, E. "An *Enterococcus faecium* Isolated from Bovine Feces in Italy Shares *optrA*- and *poxtA*-Carrying Plasmids with Enterococci from Switzerland" Microb Drug Resist, 2023 ;29:438-442. doi: 10.1089/mdr.2023.0055. Epub 2023 Jul 31.

J4. Cinthi, M., Massacci, F.R., Coccitto, S.N., Albini, E., Cucco, L., Orsini, M., Simoni, S., Giovanetti, E., Brenciani, A., Magistrali, C.F. "Characterization of a prophage and a defective integrative conjugative element carrying the *optrA* gene in linezolid-resistant *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from pigs, Italy" J Antimicrob Chemother, 2023 ;78:1740-1747. doi: 10.1093/jac/dkad164.

J5. Coccitto, S.N., Cinthi, M., Simoni, S., Vignaroli, C., Massacci, F.R., Albini, E., Garofalo, C., Aquilanti, L., Magistrali, C.F., Brenciani, A., Giovanetti, E. "Identification of plasmids co-carrying *cfr*(D)/*optrA* and *cfr*(D2)/*poxtA* linezolid resistance genes in two *Enterococcus avium* isolates from swine brain" Vet Microbiology, doi:10.1016/j.vetmic.2023.109749.

J6. Cinthi, M., Coccitto, S.N., Simoni, S., Zeni, G., Mazzariol A., Pocognoli, A., Xiang-Dang Du, Brenciani, A., Giovanetti, E. "Characterization of a defective *erm*(T) gene in a blood *Enterococcus faecium* strain . Publication status: accepted

J7. Cinthi, M., Coccitto, S.N., Pocognoli, A., Zeni, G., Mazzariol, A., Di Gregorio, A., Vignaroli, C., Brenciani, A., Giovanetti, E. "Persistence and evolution of linezolid- and methicillin-resistant *Staphylococcus epidermidis* ST2 and ST5 clones in an Italian hospital". Publication status: submitted

J8. Coccitto, N.S., Cinthi, M., Simoni, S., Pocognoli, A., Zeni, G., Mazzariol, A., Morroni, G., Mingoia, M., Giovanetti, E., Brenciani, A.,Vignaroli, C. "Genetic analysis of vancomycin-variable *Enterococcus faecium* clinical isolates in Italy". Publication status: submitted

J9. Brenciani, A., Cinthi, M., Coccitto, S., Massacci, F.R., Albini, E., Cucco, L., Paniccià, M., Freitas, A. R., Schwarz, S., Giovanetti, E., Magistrali, C. "Global spread of the linezolid-resistant Enterococcus faecalis optrA ST476 clonal lineage". Publication status: submitted

List of publications on conference proceedings

C1. ...

C2. ...

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

B1. ...

B2. ...

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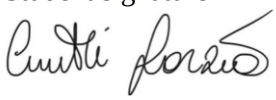

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