Assessment of the spread of florfenicol resistant enterococci harboring oxazolidinone resistance genes – a "One Health Approach"

Background:
Linezolid is a highly effective last-resort antibiotic used for the treatment of infections caused by multi-drug resistant Gram-positive pathogens such as vancomycin-resistant enterococci. Linezolid belongs to the oxazolidinone antibiotics, which inhibit protein synthesis by binding to the 23S ribosomal RNA of the 50S subunit. Linezolid resistance mechanisms include mutations in the 23S rRNA binding site, mutations in the genes encoding ribosomal proteins L3, L4, or L22, and the acquisition of the transferable genes *cfr*, *optrA*, or *poxtA.*

WP1: Faecal carriage of enterococci harbouring oxazolidinone resistance genes in the community in Switzerland

Hypothesis: Healthy humans are already carriers of cfr$^+$, optrA$^+$ and poxtA$^+$ enterococci. If so, might there be a foodborne link?

A total of 399 stool samples from healthy individuals were cultured on a selective medium containing 10 mg/L florfenicol. Resulting enterococci were screened by PCR for the presence of *cfr*, *optrA* and *poxtA*. A hybrid approach combining short-read and long-read whole genome sequencing was used to analyse the genetic context of the *cfr*, *optrA* and *poxtA* genes. *E. faecalis* (n=6), *E. faecium* (n=6), *E. gallinarum* (n=1), and *E. hirae* (n=2) were detected in 15/399 (3.8%) of the faecal samples. They carried *cfr*+*poxtA*, *optrA*, *optrA*+*poxtA*, or *poxtA*. In most *optrA*-positive isolates, the genetic environments of *optrA* were highly variable but often resembled previously described platforms. In most *poxtA*$^-$positive isolates, the *poxtA* gene was flanked on both sides by IS1216E elements and located on medium sized plasmids.
Conclusions: Faecal carriage of *cfr, optrA* and *poxtA* harbouring *Enterococcus* spp. in healthy humans demonstrates the possibility of spread of oxazolidinone resistance genes into the community. Given the importance of linezolid as a last resort antibiotic for the treatment of serious infections caused by Gram-positive pathogens, the detection of the oxazolidinone resistance determinants in enterococci from healthy humans is of concern for public health.

- Nüesch-Inderbinen et al. (2022). Faecal carriage of enterococci harbouring oxazolidinone resistance genes among employees of food processing plants in Switzerland. JAC, 77(10):2779-2783. doi:10.1093/jac/dkac260

WP2: Dissemination of florfenicol resistant enterococci harboring oxazolidinone resistance genes in the environment

Hypothesis: The aquatic ecosystem contributes to the dissemination of linezolid-resistant enterococci.

River water
Ten florfenicol resistant *Enterococcus* spp. isolates recovered in 2020 from streams of different regions in Switzerland were genetically characterized in this study. All isolates underwent short-read sequencing (Illumina MiniSeq), and seven isolates with suspected plasmid-encoded linezolid-resistance determinants additionally underwent long-read sequencing (MinION, ONT). The 10 florfenicol-resistant enterococci isolates all carried phenicol-oxazolidinone-resistance genes, i.e., *optrA* (3x *E. faecalis*, 2x *E. faecium*, 2x *E. hirae*, 1x *E. raffinosus*), *poxtA* (1x *E. faecium*), or both (1x *E. faecium*). Most isolates were also non-susceptible to linezolid.
In most genomes, *optrA* and *poxtA* were embedded in transposition units integrated into plasmids or into the chromosomal *radC*. For the first time a chromosomally integrated *optrA* in an *Enterococcus raffinosus* isolate is described.
Conclusion: Our findings suggest that the aquatic ecosystem contributes to the dissemination of linezolid-resistant enterococci which is a worrisome aspect from a public health perspective.

- Biggel et al. (2021). Genetic context of *optrA* and *poxtA* in florfenicol-resistant enterococci isolated from flowing surface water in Switzerland. Antimicrobial Agents and Chemotherapy 65:e01083-21. https://doi.org/10.1128/AAC.01083-21

- Nüesch-Inderbinen et al. (2021), M. Linezolid-resistant *Enterococcus faecalis* ST16 harbouring *optrA* on a Tn6674-like element isolated from surface water. Journal of Global Antimicrobial Resistance 25, 89–92. https://doi.org/10.1016/j.jgar.2021.02.029.

WP3: Farm animals as a potential reservoir of florfenicol resistant enterococci harboring oxazolidinone resistance genes in Switzerland

Hypothesis: The use of florfenicol and other antibiotics in farm animals may co-select enterococci that carry acquired transferable resistance genes which confer resistance to linezolid.

Fattening pigs at slaughter

A total of 31 florfenicol-resistant enterococcal isolates were obtained from 27 (5%) of 565 fecal samples of fattening pigs from seven (11%) of 62 farms. Screening by PCR for the presence of the oxazolidinone resistance genes revealed the presence of *cfr/poxtA* in 1/31, *optrA* in 15/31 and *poxtA* in 15/31 enterococcal isolates, respectively. WGS analysis of ten selected isolates (one *Enterococcus* species per herd) showed the presence of *E. faecalis* (n=1), *E. faecium* (n=1), and *E. hirae* (n=1), harboring *optrA18*, *optrA7* and a new *optrA* allele, respectively. *E. durans* (n=1), *E. faecium* (n=4), and *E. hirae* (n=1), carried the wild-type *poxtA*, and *E. faecalis* (n=1) co-harbored *cfr(D)* and *poxtA2*. With the exception of *optrA7*, all oxazolidinone resistance genes were found on plasmids. MLST analysis identified *E. faecalis* ST19 and ST376, *E. faecium* ST80 belonging to hospital-adapted clade A1, and *E. faecium* ST21, ST55, ST269 and ST416, belonging to clade A2 which represents human commensals and animal strains.

Conclusion: The occurrence of oxazolidinone resistance determinants in porcine enterococci including genotypes belonging to major human pathogens is concerning. Cross-selection through the use of florfenicol in animal farming has emerged as a problem that needs to be addressed in order to mitigate the dissemination to humans, especially through the food chain.

- Nüesch-Inderbinen et al. (2022). Fattening pigs are a reservoir of florfenicol resistant enterococci harboring oxazolidinone resistance genes. Journal of Food Protection 85(5):740-746. doi: 10.4315/JFP-21-431

Cattle at slaughter

A total of 618 cecal samples taken from beef cattle and veal calves at slaughter originating from 199 herds were cultured after an enrichment step on a selective medium containing 10 mg/L florfenicol. Isolates were screened by PCR for *cfr*, *optrA* and *poxtA.* Overall, 105 florfenicol-resistant isolates were obtained from 99 (16%) of the samples, corresponding to 4% of the beef cattle herds and 24% of the veal calf herds. Screening by PCR revealed the presence of *optrA* in 95 (90%), and *poxtA* in 22 (21%) of the isolates. None of the isolates contained *cfr*. Isolates included for AST and WGS analysis were *Enterococcus* (*E.*) *faecalis* (n=14), *E. faecium* (n=12), *E. dispar* (n=1), *E. durans* (n=2), and *E. gallinarum* (n=1), *Vagococcus* (*V.*) *lutrae* (n=2), *Aerococcus* (*A.*) *urinaeequi* (n=1), and *Companilactobacillus* (*C.*) *farciminis* (n=1). Thirteen isolates exhibited phenotypic linezolid resistance. Three novel OptrA variants were identified. Multilocus sequence typing identified four *E. faecium* ST18 belonging to hospital-associated clade A1. There was a difference in the replicon profile among *optrA* and *poxtA* harboring plasmids, with rep9(RepA_N) plasmids dominating in *optrA*-harboring *E. faecalis* and rep2(Inc18) and rep29(Rep_3) plasmids in *poxtA*-carrying *E. faecium.*

Conclusion: Beef cattle and veal calves are reservoirs for enterococci with acquired linezolid resistance genes *optrA* and *poxtA*. The presence of *E. faecium* ST18 highlights the zoonotic potential of some bovine isolates. The dispersal of clinically relevant oxazolidinone resistance genes throughout a wide variety of species including *Enterococcus* spp., *V. lutrae, A. urinaeequi* and the probiotic *C. farciminis* in food-producing animals is a public health concern.

- Nüesch-Inderbinen et al. (2023). Frontiers in Microbiology 14:1150070. doi: 10.3389/fmicb.2023.1150070.

Poultry at slaughter
In total 100 fecal samples of chicken were collected at slaughterhouse level from the crates of 100 poultry flocks (87 BTS label farms, 8 free range farms, 5 Bio label farms) distributed throughout Switzerland. Florfenicol resistant Staphylococci and Enterococci were selected after an enrichment step in BHI broth with 6.5% NaCl on Bile Aesculin Acid plates supplemented with 10 mg/L florfenicol. The species identification was carried out by MALDI-TOF, and the resistance genes were detected by polymerase chain reaction.
The prevalence of florfenicol resistant Staphylococci and Enterococci was 19% (mainly *M. sciuri*) and 0% respectively. Only one florfenicol resistant *Mammaliicoccus sciuri* harbouring *cfrA* on a 15kb plasmid was detected. This plasmid encoded additionally *erm*(B), *tet*(L) and *lsa*(B). Moreover, the strain harboured *mecA1*, *sal(A)*, *fexA* on the chromosom.
Conclusion: So far, there is a very low occurrence of florfenicol resistant staphylococci and enterococci harboring oxazolidinone resistance genes in poultry flocks in Switzerland.

- Heyvaert, L. (2022). student project

WP4: Pets as a potential reservoir of florfenicol resistant enterococci harboring oxazolidinone resistance genes in Switzerland

Project ongoing

WP5: Potential spread of florfenicol resistant enterococci harboring oxazolidinone resistance genes along the food and feed chain

Hypothesis: Food products can be vectors of florfenicol resistant enterococci harboring oxazolidinone resistance genes. However, due to a lack of data a risk assessment for different food products is not yet possible.

Swiss alpine hard cheese made with unpasteurized cow's milk
The aim of this study was to provide further microbiological data for Swiss alpine hard cheese made using raw cow's milk. A total of 100 cheese samples were collected between July 2021 and January 2022 from dairies, cheese shops, supermarkets or directly on alps throughout Switzerland and were tested for the occurrence of foodborne pathogens (*Salmonella*, *L. monocytogenes*, STEC). Since raw milk can also introduce antimicrobial resistant bacteria in the cheese making process, all samples were further tested for the occurrence of florfenicol-resistant enterococci harbouring transferable resistance genes *cfr*, *optrA*, and/or *poxtA*. Florfenicol-resistant enterococci were not detected in any of the 100 cheese samples.
Conclusion: Swiss alpine hard cheese does not represent a relevant risk for the transfer of florfenicol resistant enterococci harboring oxazolidinone resistance genes along the foodchain.

- Beth, A. (2022). vet. med. master thesis

Hamburger patties and beef tartare samples
In total 106 raw hamburger patties and tartare samples were collected. Florfenicol resistant Staphylococci and Enterococci were selected after an enrichment step in BHI broth with 6.5% NaCl on Bile Aesculin Acid plates supplemented with 10 mg/L florfenicol. The species identification was carried out by MALDI-TOF, and the resistance genes were detected by polymerase chain reaction. The prevalence of florfenicol resistant Staphylococci and Enterococci was 38% (n=38; mainly *M. sciuri*) and 0% respectively. In four samples florfenicol resistant *Aerococcus urinaeequi* or *Aerococcus viridans* were found.
Six florfenicol resistant *Mammaliicoccus sciuri* harboued *cfrA and/or optrA* and one *Staphylococcus lentus* harboured *optrA*. Two of the three *Aerococcus viridans* isolates and one *Aerococcus urinaeequi* isolate harboured an *optrA* gene.

Conclusion: Raw hamburger patties and beef tartare does not represent a relevant risk for the transfer of florfenicol resistant enterococci harboring oxazolidinone resistance genes along the foodchain.

- Heyvaert, L. (2022). student project

Pork meat

In total 50 raw pork meat samples (meat origin Switzerland) were collected between May and June 2023 at retail level in Switzerland. Florfenicol resistant Staphylococci and Enterococci were selected after an enrichment step in BHI broth with 6.5% NaCl on Bile Aesculin Acid plates supplemented with 10 mg/L florfenicol. The species identification was carried out by MALDI-TOF, and the resistance genes were detected by sequencing the isolates. The prevalence of florfenicol resistant Staphylococci and Enterococci was 2% (n=1; *M. sciuri*) and 0% respectively The florfenicol resistant *Mammaliicoccus sciuri* harboued *fexA.*

Conclusion: Raw pork meat does not represent a relevant risk for the transfer of florfenicol resistant enterococci harboring oxazolidinone resistance genes along the foodchain.

- Tan, J. (2023). student project

Salami type sausages

We collected 98 raw-meat sausages (salami type sausages) on the retail level in Switzerland in March 2023. An amount of 10 g was homogenized in 90 mL brain heart infusion broth with 6% NaCl and incubated for 18–24 h at 37°C. Enriched samples were streaked on bile esculin azide agar supplemented with 10 mg/L florfenicol and incubated at 37°C for 48 h. Presumptive *Enterococci* colonies were recovered from one sausage (Finocchiona). The genome of *Enterococcus faecalis* 90_2023 consists of a 2,748,665 bp chromosome, the 92,098 bp plasmid p90_2023_A, and the 62,024 bp plasmid p90_2023_B. The isolate belongs to *E. faecalis* cgMSLT complex type 3262 and harbors 16 antimicrobial resistance genes, including *cat*A8, *fexA*, and a truncated *optrA* gene on a RepA_N plasmi

Conclusion: Salami type sausages may represent a risk for the transfer of florfenicol resistant enterococci harboring oxazolidinone resistance genes along the foodchain.

- Kelbert et al. (2023). Microbiology Resource Announcements 12(10):e0061023. doi: 10.1128/MRA.00610-23.

Raw meat-based diets (RMBDs) for companion animals

A total of 59 samples of different types of RMBDs from ten different suppliers were screened for florfenicol resistant Gram-positive bacteria using a selective culture medium. Isolates were phenotypically and genotypically characterised. A total of 27 *Enterococcus faecalis*, *Enterococcus faecium*, and *Vagococcus lutrae* were found in 24 (41%) of the samples. The *optrA* gene was identified in 24 (89%) isolates, and a total of 11 different OptrA variants were determined. The *poxtA* gene was found in six (22%), and the *cfr* gene was found in five (19%) isolates, respectively.

The minimum inhibitory concentrations of chloramphenicol and linezolid ranged from 24 mg/L–256 mg/L, and 1.5 mg/L–8 mg/L, respectively. MLST analysis of the 17 *E. faecalis* identified ten different STs, with ST593 (n=4) and ST207 (n=2) occurring more than once, and two isolates assigned to novel STs. *E. faecium* isolates were assigned to four different STs (168, 264, 822, and 1846).

Conclusion: The high occurrence of Gram-positive bacteria harbouring genes encoding resistance to the critically important linzeolid is a matter of concern since such bacteria could easily spread from companion animals to humans with close contact with their animals.

- Nüesch-Inderbinen et al. (2023). Eurosurveillance, 28(6):pii=2200496. https://doi.org/10.2807/1560-7917.ES.2023.28.6.2200496

Further projects on different other food products are ongoing….

- Herbs and ready-to-eat salads