J Antimicrob Chemother 2022; **77**: 49–57
https://doi.org/10.1093/jac/dkab376 Advance Access publication 21 October 2021

Journal of Antimicrobial Chemotherapy

Large diversity of linezolid-resistant isolates discovered in food-producing animals through linezolid selective monitoring in Belgium in 2019

Michaël Timmermans[1,2], Bert Bogaerts[3], Kevin Vanneste[3], Sigrid C. J. De Keersmaecker[3], Nancy H. C. Roosens[3], Carole Kowalewicz[1], Guillaume Simon[1], Maria A. Argudín[4]†, Ariane Deplano[4,5], Marie Hallin[4,5,6], Pierre Wattiau[1], David Fretin[1], Olivier Denis[6,7] and Cécile Boland[1]*

[1]Veterinary Bacteriology, Sciensano, Ixelles, Belgium; [2]Faculté de Médecine, Université Libre de Bruxelles, Brussels, Belgium; [3]Transversal Activities in Applied Genomics, Sciensano, Ixelles, Belgium; [4]National Reference Centre-Staphylococcus aureus, Department of Microbiology, Hôpital Erasme, Université Libre de Bruxelles, Brussels, Belgium; [5]Department of Microbiology, LHUB-ULB, Université Libre de Bruxelles, Brussels, Belgium; [6]Ecole de Santé Publique, Université Libre de Bruxelles, Brussels, Belgium; [7]Laboratory of Clinical Microbiology, National Reference Center for Monitoring Antimicrobial Resistance in Gram-Negative Bacteria, CHU UCL Namur, Yvoir, Belgium

*Corresponding author. E-mail: cecile.boland@sciensano.be
†Present address: Molecular Biology Laboratory, Tour R. Franklin, Cliniques Universitaires Saint Luc UCL, Brussels, Belgium.

Received 20 April 2021; accepted 20 September 2021

Background: Linezolid is a critically important antibiotic used to treat human infections caused by MRSA and VRE. While linezolid is not licensed for food-producing animals, linezolid-resistant (LR) isolates have been reported in European countries, including Belgium.

Objectives: To: (i) assess LR occurrence in staphylococci and enterococci isolated from different Belgian food-producing animals in 2019 through selective monitoring; and (ii) investigate the genomes and relatedness of these isolates.

Methods: Faecal samples ($n = 1325$) and nasal swab samples ($n = 148$) were analysed with a protocol designed to select LR bacteria, including a 44–48 h incubation period. The presence of LR chromosomal mutations, transferable LR genes and their genetic organizations and other resistance genes, as well as LR isolate relatedness (from this study and the NCBI database) were assessed through WGS.

Results: The LR rate differed widely between animal host species, with the highest rates occurring in nasal samples from pigs and sows (25.7% and 20.5%, respectively) and faecal samples from veal calves (16.4%). WGS results showed that LR determinants are present in a large diversity of isolates circulating in the agricultural sector, with some isolates closely related to human isolates, posing a human health risk.

Conclusions: LR dedicated monitoring with WGS analysis could help to better understand the spread of LR. Cross-selection of LR transferable genes through other antibiotic use should be considered in future action plans aimed at combatting antimicrobial resistance and in future objectives for the rational use of antibiotics in a One Health perspective.

Introduction

Linezolid is an antibiotic of the oxazolidinone family used as a critically important antibiotic to treat MRSA and VRE infections in humans.[1] Linezolid resistance (LR) can be caused by point mutations in the 23S rRNA gene (mainly G2576T and G2505A) or through acquisition of *cfr*, *optrA* or *poxtA*, often located on plasmids.[2–5] *cfr* was discovered in 2000 in a *Staphylococcus sciuri* calf isolate[6] and confers cross-resistance to phenicols, lincosamides, linezolid, pleuromutilins and streptogramin A, referred to as a $PhLOPS_A$ phenotype.[3] *cfr* codes for a methyltransferase that modifies position A2503 of the 23S rRNA.[3] *optrA* codes for an ARE ABC-F protein and was first described in China in *Enterococcus faecalis* and *Enterococcus faecium* isolates of human and animal

origins.[4,7] *optrA* confers resistance to oxazolidinones and phenicols.[4] *poxtA* codes for an ARE ABC-F protein and was discovered in Italy in 2018 in an MRSA of clinical origin from 2015.[5] It confers reduced susceptibility to oxazolidinones, phenicols and tetracyclines.[5] All three genes give cross-resistance to linezolid and phenicols and have already been found with phenicol resistance genes like *fexA* and *fexB*.[4,8]

Worldwide emergence of LR bacteria like MRSA and VRE has been described in recent studies. In Belgium, the first clinical case associated with *cfr* was reported in 2017,[9] while a study from 2019 reported the presence of *optrA* in clinical isolates collected in 2014.[10] In food-producing animals, LR isolates were reported in various European countries,[11] including Belgium through its antimicrobial resistance (AMR) official monitoring (OM),[12] despite linezolid not being licensed for this sector,[13] highlighting the importance of monitoring LR with a One Health approach.

This study aims to: (i) assess LR occurrence in staphylococci and enterococci isolated from food-producing animals through dedicated selective monitoring conducted for the first time in Belgium; and (ii) investigate the genomes and relatedness of LR isolates.

Materials and methods

Isolate collection and identification

A total of 1325 faecal samples (broilers $n = 295$, turkeys $n = 86$, laying hens $n = 205$, breeding hens $n = 163$, veal calves $n = 293$ and pigs $n = 283$) and 148 nasal swab samples (sows $n = 78$ and fattening pigs $n = 70$) were collected in 2019 from healthy animals and analysed at the Belgian National Reference Laboratory for AMR in MRSA and enterococci from animals. After OM analysis[12] of these samples, phenotypic LR selection was performed by collecting all bacteria grown on Petri dishes from the OM and spreading them on Columbia Sheep Blood (CSB) supplemented with linezolid (4 mg/L) (CSB-LZD). CSB-LZD plates were incubated at 37°C for 44–48 h as recommended by other studies.[14,15] Growth on CSB-LZD plates was considered positive for LR occurrence assessment. For each positive sample, two colonies were isolated and incubated on a second CSB-LZD plate for 44–48 h at 37°C and isolates were identified using MALDI-TOF MS. Only one of the two isolates was kept for each sample except if two different species were identified. Isolates were conserved at −80°C as glycerol (50%, v/v) stocks derived from overnight cultures in brain heart infusion. Samples for which two different bacterial species were found were considered as one positive case for the occurrence assessment. The collection was extended with three *Staphylococcus aureus* pig isolates from earlier OM and all isolates collected from previous studies[9,10] from human infections with a linezolid MIC_{24h} (MIC after 18–24 h of incubation) >4 mg/L, namely *E. faecalis* ($n = 3$), *E. faecium* ($n = 1$) and *S. aureus* ($n = 1$), for WGS analysis.

Antimicrobial susceptibility testing

MICs of linezolid and chloramphenicol were determined using broth microdilution (BMD) on EUVENC plates for all isolates (Sensititre™, ThermoFisher SCIENTIFIC, Waltham, USA).[12] The plates were read with the VIZION TREK instrument (ThermoFisher SCIENTIFIC) after 18–24 h of incubation (MIC_{24h}) using sensivision software (MCS diagnostics, Swalmen, Netherlands). MICs were interpreted according to the clinical breakpoints from 2020[16] (for linezolid) or EUCAST epidemiological cut-offs[17] if no clinical breakpoints were available (for chloramphenicol). MIC_{24h}s were compared with measures after 24 h of additional incubation (MIC_{48h}) to assess whether any differences would be observed (taking account of *cfr* inducibility and recommendations of previous studies).[6,15]

WGS analysis

Genomic DNA was extracted using the DNeasy® Blood and Tissue kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA purity and concentration were assessed with the Nanodrop 1000 (Isogen LifeScience, Utrecht, Netherlands). Isolate sequencing libraries were created using Nextera XT DNA library preparation (Illumina, San Diego, CA, USA) according to the manufacturer's instructions and subsequently sequenced using MiSeq V3 chemistry (Illumina) for the production of 2×250 bp paired-end reads. All sequencing data have been submitted to SRA under BioProject PRJNA670413.[18] Reads were trimmed and *de novo* assembled as described by Bogaerts *et al.*[19] Ribosomal MLST (rMLST) was used to confirm the species of each isolate using a threshold of >75% matching loci,[20] using the methodology for sequence typing as described previously.[19] Detection of LR genes was performed as described for gene detection by Bogaerts *et al.*[19] using the sequences from the LRE-Finder database.[21] Hits with ≤90% sequence identity or ≤90% target coverage were removed. The same methodology was used to detect other AMR genes using the ResFinder database.[22] A local installation of the LRE-Finder tool (checked out from BitBucket on 8 August 2020), was used with default settings to detect 23S rRNA mutations. *E. faecium*, *E. faecalis* and *S. aureus* isolates were typed via core-genome MLST (cgMLST) as described previously.[19] Read mapping was executed with BWA-MEM with default settings and visualized with Tablet (version 1– 19.09.03).[23] Genetic organizations were determined using Blast analysis. Classic MLST schemes were retrieved from PubMLST.org,[24] while cgMLST schemes were retrieved from cgMLST.org for *E. faecalis* and *E. faecium*[25,26] and from PubMLST.org for *S. aureus*. Novel alleles and STs for classic MLST were submitted to PubMLST.org and novel cgMLST alleles were assigned an internal identifier before starting the phylogenomic analysis.

Phylogenomic analysis

Relatedness between isolates was determined by constructing phylogenies based on cgMLST results for *E. faecium*, *E. faecalis* and *S. aureus*. Allele matrices were filtered by removing isolates with ≤90% of loci detected and afterwards removing loci detected in ≤90% of isolates. Minimum spanning trees (MSTs) were constructed from the filtered allele matrices using GrapeTree 1.5.0 with the 'method' option set to 'MSTreeV2'.[27] Phylogenies were visualized and annotated in IToL.[28] The wider phylogenomic context of isolates was investigated as follows. Classic MLST was performed on all available genomes in the NCBI Assembly database that were also present in RefSeq for *E. faecalis* ($n = 1423$), *E. faecium* ($n = 1972$) and *S. aureus* ($n = 11\,945$) (retrieved in October 2020) using the methodology described above. Isolates were then grouped by ST and separate MSTs were constructed for each group containing at least one isolate from this study and five isolates in total. SNP phylogenies were constructed to zoom in on certain groups. Read data for NCBI samples were retrieved through the ENA portal API, only retaining isolates with paired-end Illumina data and at least 20 000 reads available. The CFSAN SNP pipeline 2.0.2 was used with default parameters to construct SNP matrices.[29] MEGA-Computing Core 10.0.4 was then used to detect the best evolutionary model and construct maximum-likelihood phylogenetic trees based on the preserved SNP matrices,[30] as described previously.[31] The least fragmented genome within an ST cluster was selected as the reference genome for read mapping. SNP addresses were determined using SnapperDB 1.0.6[32] and PHEnix v1.4.1 for all isolates included in the SNP phylogenies using the same reference genome as for the SNP matrices, as described previously.[33] This SNP address is a strain-level 7-digit nomenclature based on the number of pair-wise SNP differences. Each digit represents the cluster membership for the given number of SNP differences, starting (right to left) with 0 (e.g. no SNP differences) to 5, 10, 25, 50, 100 and 250. Isolates sharing the same cluster digit differ by fewer than the corresponding number of SNPs.

Table 1. LR gene profiles observed in this study

LR gene profile	Bacterial species (host origin and number of isolates) of isolates
cfr	*S. aureus* (pig *n* = 5, human *n* = 1), *S. sciuri* (pig *n* = 1)
optrA	*E. faecalis* (poultry *n* = 9, pig *n* = 22, cattle *n* = 39, human *n* = 3), *E. faecium* (poultry *n* = 3, pig *n* = 8, cattle *n* = 3, human *n* = 1), *E. hirae* (pig *n* = 7), *E. casseliflavus* (pig *n* = 4), *E. gallinarum* (pig *n* = 2, cattle *n* = 1), *E. asini* (pig *n* = 1), *E. saccharolyticus* (pig *n* = 1), *E. durans* (cattle *n* = 1)
poxtA	*E. faecium* (poultry *n* = 8, pig *n* = 11), *P. pentosaceus* (pig *n* = 1)
cfr, optrA	*E. gallinarum* (poultry *n* = 1, pig *n* = 1)
optrA, poxtA	*E. faecium* (poultry *n* = 3, pig *n* = 4, cattle *n* = 6), *E. faecalis* (pig *n* = 3)
cfr, optrA, poxtA	*E. faecalis* (pig *n* = 1)

Geographical clustering

K-means clustering using the 'kmeans' function in R 3.6.1 with the 'centers' parameter set to six was performed on the coordinates of the samples to divide locations into six geographically related groups. The raw coordinates could not be shared due to privacy considerations, but each unique set of coordinates was randomly assigned a number. Location and geographical cluster numbers are provided as annotations in the phylogenetic trees.

Results

Linezolid selective monitoring and collection of isolates

A total of 105/1325 (7.9%) faecal samples [0/86 from turkeys (0%), 2/205 from laying hens (1.0%), 14/295 from broilers (4.7%), 10/163 from breeding hens (6.1%), 31/283 from pigs (11.0%) and 48/293 from veal calves (16.4%)] and 34/148 (23.0%) nasal samples [16/78 from sows (20.5%) and 18/70 from fattening pigs (25.7%)] exhibited growing bacteria on CSB-LZD plates after 48 h of incubation. Among these 139 samples, 147 isolates were collected and stored: 77 *E. faecalis*, 47 *E. faecium*, 7 *Enterococcus hirae*, 5 *Enterococcus gallinarum*, 4 *Enterococcus casseliflavus*, 1 *Enterococcus asini*, 1 *Enterococcus durans*, 1 *Enterococcus saccharolyticus*, 2 *S. aureus*, 1 *S. sciuri* and 1 *Pediococcus pentosaceus* (kept for this study) (MALDI-TOF MS identification; Table S1, available as Supplementary data at *JAC* Online).

Incubation comparison of BMD plates after 24 and 48 h

After 24 h, 118/147 isolates (80.3%) were above the linezolid breakpoint, while all 147 isolates were above the breakpoint after 48 h. Chloramphenicol susceptibility testing showed that 50 isolates were susceptible to this antibiotic after 24 h, of which 13 remained under the thresholds after 48 h (Table S1).

Genetic characterization

Among the 147 isolates from 2019 and the 8 added isolates (3 *E. faecalis*, 1 *E. faecium* and 4 *S. aureus*), all but 4 harboured *cfr* (*n* = 7, 1 *S. sciuri* and 6 *S. aureus*), *optrA* (*n* = 105, 1 *E. asini*, 1 *E. saccharolyticus*, 1 *E. durans*, 3 *E. gallinarum*, 4 *E. casseliflavus*, 7 *E. hirae*, 15 *E. faecium* and 73 *E. faecalis*), *poxtA* (*n* = 20, 1 *P. pentosaceus* and 19 *E. faecium*), *cfr* and *optrA* (*n* = 2, 2 *E. gallinarum*), *optrA* and *poxtA* (*n* = 16, 3 *E. faecalis* and 13 *E. faecium*) or all three (*n* = 1, 1 *E. faecalis*) (Table 1). When multiple LR genes were present, they were located on different contigs except for the co-occurrences of *cfr* and *optrA*. The four isolates lacking LR genes contained 23sRNA mutations: G2505A (*n* = 1, 1 *E. faecalis*) and G2576T (*n* = 3, 1 *E. faecium* and 2 *E. faecalis*). Detected alleles for *cfr, optrA* and *poxtA* and mutations in 23sRNA are listed in Table S2.

Of the 37 isolates carrying *poxtA*, 36 also carried *fexB*, but never on the same contig. A read mapping of these 36 isolates on plasmid pE1077-23 (GenBank: MT074684, isolated from an *E. faecium* strain from swine in China and carrying both *poxtA* and *fexB*) mapped the region shown in Figure S1 in all cases, suggesting that the isolates could carry this genetic organization.

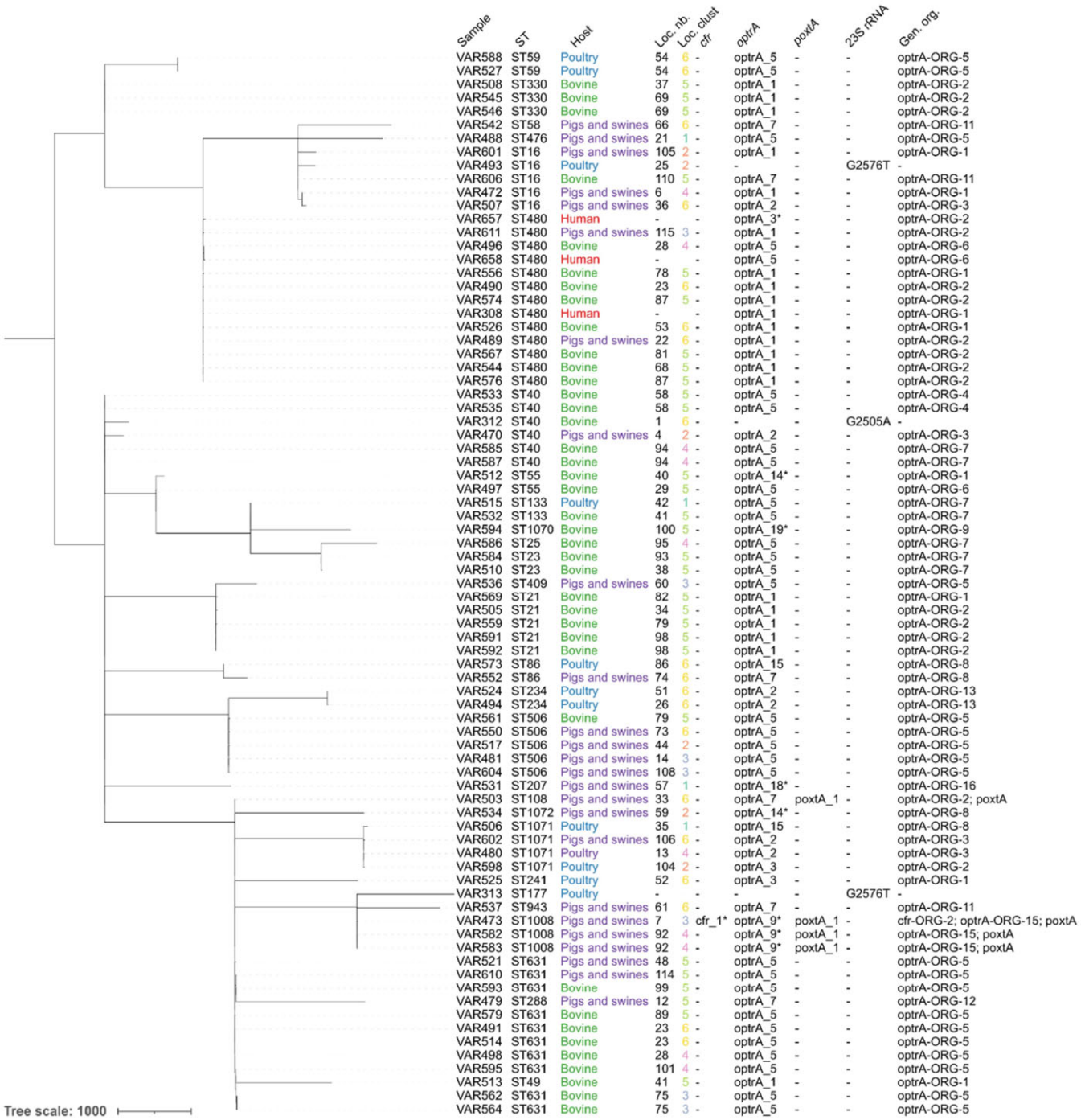
Out of the 10 isolates carrying *cfr*, 8 also carried *fexA* on the same contig (Table S2 and Figure S2). Four genetic organizations were observed, called here *cfr*-ORG-1 (*n* = 7), *cfr*-ORG-2 (*n* = 1), *cfr*-ORG-3 (*n* = 1) and *cfr*-ORG-4 (*n* = 1) (Figure S2). In each organization, *cfr* was associated with different resistance genes: *fexA* (*cfr*-ORG-1), *lnuE* (*cfr*-ORG-2), *aad-D-2, aph(2'')-Ic, ble* and *optrA* (*cfr*-ORG-3) and *aad-D-2, aph(2'')-Ic, ble, erm(B), optrA* and *fexA* (*cfr*-ORG-4).

For *optrA*, 20 genetic organizations were observed (Figure S3), excluding *cfr* organizations also carrying *optrA* (*n* = 2). Contigs carrying *optrA* also contained *fexA* in 104/124 isolates and/or *erm(A)* in 47/124, *ant(9)-Ia* in 38/124, *erm(B)* in 6/124, *tetL, tetM, aph(2'')* and/or *aadD* in 2/124 and/or *aac(6')-aph(2'), ant(6)-Ia, lnu(B)* and/or *lsa(E)* in 1/124.

Species identification with rMLST succeeded for 151/155 isolates and was consistent with MALDI-TOF MS identifications. Three isolates could only be identified up to the *Enterococcus* genus (VAR314, VAR522 and VAR530) and VAR572 (*E. asini*) was not identified through rMLST. Since these four isolates were classified by MALDI-TOF MS as species for which only a very limited number of rMLST profiles were available in the rMLST database, we hypothesize that the detection failed due to the presence of novel rMLST alleles not yet present in the underlying rMLST database.

Phylogenomic analysis

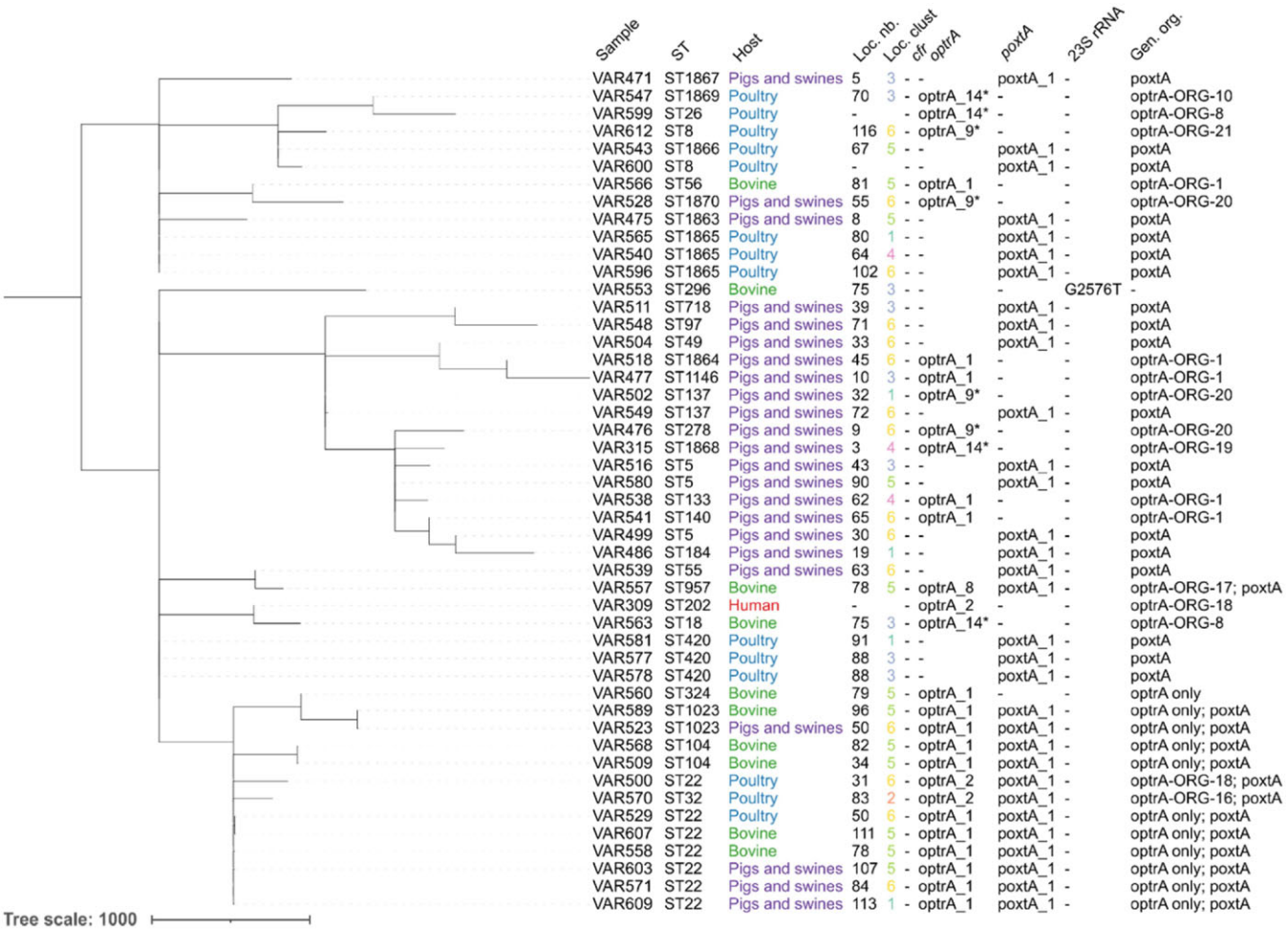
Except for the *E. faecalis* VAR492 isolate, more than 90% of cgMLST loci were detected in all *E. faecalis, E. faecium* and *S. aureus* isolates from this study. This resulted in phylogenetic trees with 79, 48 and 6 isolates, respectively (Figures 1–3). For both *Enterococcus* species, very large phylogenetic differences were observed, along with some smaller clades containing more closely related isolates. In total, 28 (including 3 new) and 32 (including 8



Sample	ST	Host	Loc. nb.	Loc. clust	cfr	optrA	poxtA	23S rRNA	Gen. org.
VAR588	ST59	Poultry	54	6	-	optrA_5	-	-	optrA-ORG-5
VAR527	ST59	Poultry	54	6	-	optrA_5	-	-	optrA-ORG-5
VAR508	ST330	Bovine	37	5	-	optrA_1	-	-	optrA-ORG-2
VAR545	ST330	Bovine	69	5	-	optrA_1	-	-	optrA-ORG-2
VAR546	ST330	Bovine	69	5	-	optrA_1	-	-	optrA-ORG-2
VAR542	ST58	Pigs and swines	66	6	-	optrA_7	-	-	optrA-ORG-11
VAR488	ST476	Pigs and swines	21	1	-	optrA_5	-	-	optrA-ORG-5
VAR601	ST16	Pigs and swines	105	2	-	optrA_1	-	-	optrA-ORG-1
VAR493	ST16	Poultry	25	2	-	-	-	G2576T	-
VAR606	ST16	Bovine	110	5	-	optrA_7	-	-	optrA-ORG-11
VAR472	ST16	Pigs and swines	6	4	-	optrA_1	-	-	optrA-ORG-1
VAR507	ST16	Pigs and swines	36	6	-	optrA_2	-	-	optrA-ORG-3
VAR657	ST480	Human	-	-	-	optrA_3*	-	-	optrA-ORG-2
VAR611	ST480	Pigs and swines	115	3	-	optrA_1	-	-	optrA-ORG-2
VAR496	ST480	Bovine	28	4	-	optrA_5	-	-	optrA-ORG-6
VAR658	ST480	Human	-	-	-	optrA_5	-	-	optrA-ORG-6
VAR556	ST480	Bovine	78	5	-	optrA_1	-	-	optrA-ORG-1
VAR490	ST480	Bovine	23	6	-	optrA_1	-	-	optrA-ORG-2
VAR574	ST480	Bovine	87	5	-	optrA_1	-	-	optrA-ORG-2
VAR308	ST480	Human	-	-	-	optrA_1	-	-	optrA-ORG-1
VAR526	ST480	Bovine	53	6	-	optrA_1	-	-	optrA-ORG-1
VAR489	ST480	Pigs and swines	22	6	-	optrA_1	-	-	optrA-ORG-2
VAR567	ST480	Bovine	81	5	-	optrA_1	-	-	optrA-ORG-2
VAR544	ST480	Bovine	68	5	-	optrA_1	-	-	optrA-ORG-2
VAR576	ST480	Bovine	87	5	-	optrA_1	-	-	optrA-ORG-2
VAR533	ST40	Bovine	58	5	-	optrA_5	-	-	optrA-ORG-4
VAR535	ST40	Bovine	58	5	-	optrA_5	-	-	optrA-ORG-4
VAR312	ST40	Bovine	1	6	-	-	-	G2505A	-
VAR470	ST40	Pigs and swines	4	2	-	optrA_2	-	-	optrA-ORG-3
VAR585	ST40	Bovine	94	4	-	optrA_5	-	-	optrA-ORG-7
VAR587	ST40	Bovine	94	4	-	optrA_5	-	-	optrA-ORG-7
VAR512	ST55	Bovine	40	5	-	optrA_14*	-	-	optrA-ORG-1
VAR497	ST55	Bovine	29	5	-	optrA_5	-	-	optrA-ORG-6
VAR515	ST133	Poultry	42	1	-	optrA_5	-	-	optrA-ORG-7
VAR532	ST133	Bovine	41	5	-	optrA_5	-	-	optrA-ORG-7
VAR594	ST1070	Bovine	100	5	-	optrA_19*	-	-	optrA-ORG-9
VAR586	ST25	Bovine	95	4	-	optrA_5	-	-	optrA-ORG-7
VAR584	ST23	Bovine	93	5	-	optrA_5	-	-	optrA-ORG-7
VAR510	ST23	Bovine	38	5	-	optrA_5	-	-	optrA-ORG-7
VAR536	ST409	Pigs and swines	60	3	-	optrA_5	-	-	optrA-ORG-5
VAR569	ST21	Bovine	82	5	-	optrA_1	-	-	optrA-ORG-1
VAR505	ST21	Bovine	34	5	-	optrA_1	-	-	optrA-ORG-2
VAR559	ST21	Bovine	79	5	-	optrA_1	-	-	optrA-ORG-2
VAR591	ST21	Bovine	98	5	-	optrA_1	-	-	optrA-ORG-2
VAR592	ST21	Bovine	98	5	-	optrA_1	-	-	optrA-ORG-2
VAR573	ST86	Poultry	86	6	-	optrA_15	-	-	optrA-ORG-8
VAR552	ST86	Pigs and swines	74	6	-	optrA_7	-	-	optrA-ORG-8
VAR524	ST234	Poultry	51	6	-	optrA_2	-	-	optrA-ORG-13
VAR494	ST234	Poultry	26	6	-	optrA_2	-	-	optrA-ORG-13
VAR561	ST506	Bovine	79	5	-	optrA_5	-	-	optrA-ORG-5
VAR550	ST506	Pigs and swines	73	6	-	optrA_5	-	-	optrA-ORG-5
VAR517	ST506	Pigs and swines	44	2	-	optrA_5	-	-	optrA-ORG-5
VAR481	ST506	Pigs and swines	14	3	-	optrA_5	-	-	optrA-ORG-5
VAR604	ST506	Pigs and swines	108	3	-	optrA_5	-	-	optrA-ORG-5
VAR531	ST207	Pigs and swines	57	1	-	optrA_18*	-	-	optrA-ORG-16
VAR503	ST108	Pigs and swines	33	6	-	optrA_7	poxtA_1	-	optrA-ORG-2; poxtA
VAR534	ST1072	Pigs and swines	59	2	-	optrA_14*	-	-	optrA-ORG-8
VAR506	ST1071	Poultry	35	1	-	optrA_15	-	-	optrA-ORG-8
VAR602	ST1071	Pigs and swines	106	6	-	optrA_2	-	-	optrA-ORG-3
VAR480	ST1071	Poultry	13	4	-	optrA_2	-	-	optrA-ORG-3
VAR598	ST1071	Poultry	104	2	-	optrA_3	-	-	optrA-ORG-2
VAR525	ST241	Poultry	52	6	-	optrA_3	-	-	optrA-ORG-1
VAR313	ST177	Poultry	-	-	-	-	-	G2576T	-
VAR537	ST943	Pigs and swines	61	6	-	optrA_7	-	-	optrA-ORG-11
VAR473	ST1008	Pigs and swines	7	3	cfr_1*	optrA_9*	poxtA_1	-	cfr-ORG-2; optrA-ORG-15; poxtA
VAR582	ST1008	Pigs and swines	92	4	-	optrA_9*	poxtA_1	-	optrA-ORG-15; poxtA
VAR583	ST1008	Pigs and swines	92	4	-	optrA_9*	poxtA_1	-	optrA-ORG-15; poxtA
VAR521	ST631	Pigs and swines	48	5	-	optrA_5	-	-	optrA-ORG-5
VAR610	ST631	Pigs and swines	114	5	-	optrA_5	-	-	optrA-ORG-5
VAR593	ST631	Bovine	99	5	-	optrA_5	-	-	optrA-ORG-5
VAR479	ST288	Pigs and swines	12	5	-	optrA_7	-	-	optrA-ORG-12
VAR579	ST631	Bovine	89	5	-	optrA_5	-	-	optrA-ORG-5
VAR491	ST631	Bovine	23	6	-	optrA_5	-	-	optrA-ORG-5
VAR514	ST631	Bovine	23	6	-	optrA_5	-	-	optrA-ORG-5
VAR498	ST631	Bovine	28	4	-	optrA_5	-	-	optrA-ORG-5
VAR595	ST631	Bovine	101	4	-	optrA_5	-	-	optrA-ORG-5
VAR513	ST49	Bovine	41	5	-	optrA_1	-	-	optrA-ORG-1
VAR562	ST631	Bovine	75	3	-	optrA_5	-	-	optrA-ORG-5
VAR564	ST631	Bovine	75	3	-	optrA_5	-	-	optrA-ORG-5

Figure 1. Phylogenetic tree containing the *E. faecalis* sequenced samples from this study. MST based on cgMLST results. Branch lengths represent the number of allele differences. Dotted lines are used to help the reader. Imperfect matches for the detected AMR alleles are indicated with an asterisk. A total of 1953 loci after filtering of the allele matrix were used to construct the phylogeny. Dashes for locations indicate that this information was not available. Dashes for genes indicate that no hits were found. Loc., location; nb., number; Gen. org., genetic organization. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

new) different STs were observed for *E. faecalis* and *E. faecium*, respectively (Figures 1 and 2 and Table S2). For the majority of observed STs, samples collected from (clinical) human cases were present in the PubMLST isolate database. Complete overviews of host statistics per species are provided in Table S3. For *S. aureus*, all six isolates were classified as ST398 and had relatively similar cgMLST profiles. For both *Enterococcus* species, samples collected at the same location generally clustered together in the phylogenies (e.g. *E. faecalis* location 54 or *E. faecium* location 88). However, for both species, samples collected in the same location with large



Sample	ST	Host	Loc. nb.	Loc. clust	cfr	optrA	poxtA	23S rRNA	Gen. org.
VAR471	ST1867	Pigs and swines	5	3	-	-	poxtA_1	-	poxtA
VAR547	ST1869	Poultry	70	3	-	optrA_14*	-	-	optrA-ORG-10
VAR599	ST26	Poultry	-		-	optrA_14*	-	-	optrA-ORG-8
VAR612	ST8	Poultry	116	6	-	optrA_9*	-	-	optrA-ORG-21
VAR543	ST1866	Poultry	67	5	-	-	poxtA_1	-	poxtA
VAR600	ST8	Poultry	-		-	-	poxtA_1	-	poxtA
VAR566	ST56	Bovine	81	5	-	optrA_1	-	-	optrA-ORG-1
VAR528	ST1870	Pigs and swines	55	6	-	optrA_9*	-	-	optrA-ORG-20
VAR475	ST1863	Pigs and swines	8	5	-	-	poxtA_1	-	poxtA
VAR565	ST1865	Poultry	80	1	-	-	poxtA_1	-	poxtA
VAR540	ST1865	Poultry	64	4	-	-	poxtA_1	-	poxtA
VAR596	ST1865	Poultry	102	6	-	-	poxtA_1	-	poxtA
VAR553	ST296	Bovine	75	3	-	-	-	G2576T	-
VAR511	ST718	Pigs and swines	39	3	-	-	poxtA_1	-	poxtA
VAR548	ST97	Pigs and swines	71	6	-	-	poxtA_1	-	poxtA
VAR504	ST49	Pigs and swines	33	6	-	-	poxtA_1	-	poxtA
VAR518	ST1864	Pigs and swines	45	6	-	optrA_1	-	-	optrA-ORG-1
VAR477	ST1146	Pigs and swines	10	3	-	optrA_1	-	-	optrA-ORG-1
VAR502	ST137	Pigs and swines	32	1	-	optrA_9*	-	-	optrA-ORG-20
VAR549	ST137	Pigs and swines	72	6	-	-	poxtA_1	-	poxtA
VAR476	ST278	Pigs and swines	9	6	-	optrA_9*	-	-	optrA-ORG-20
VAR315	ST1868	Pigs and swines	3	4	-	optrA_14*	-	-	optrA-ORG-19
VAR516	ST5	Pigs and swines	43	3	-	-	poxtA_1	-	poxtA
VAR580	ST5	Pigs and swines	90	5	-	-	poxtA_1	-	poxtA
VAR538	ST133	Pigs and swines	62	4	-	optrA_1	-	-	optrA-ORG-1
VAR541	ST140	Pigs and swines	65	6	-	optrA_1	-	-	optrA-ORG-1
VAR499	ST5	Pigs and swines	30	6	-	-	poxtA_1	-	poxtA
VAR486	ST184	Pigs and swines	19	1	-	-	poxtA_1	-	poxtA
VAR539	ST55	Pigs and swines	63	6	-	-	poxtA_1	-	poxtA
VAR557	ST957	Bovine	78	5	-	optrA_8	poxtA_1	-	optrA-ORG-17; poxtA
VAR309	ST202	Human	-		-	optrA_2	-	-	optrA-ORG-18
VAR563	ST18	Bovine	75	3	-	optrA_14*	-	-	optrA-ORG-8
VAR581	ST420	Poultry	91	1	-	-	poxtA_1	-	poxtA
VAR577	ST420	Poultry	88	3	-	-	poxtA_1	-	poxtA
VAR578	ST420	Poultry	88	3	-	-	poxtA_1	-	poxtA
VAR560	ST324	Bovine	79	5	-	optrA_1	-	-	optrA only
VAR589	ST1023	Bovine	96	5	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR523	ST1023	Pigs and swines	50	6	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR568	ST104	Bovine	82	5	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR509	ST104	Bovine	34	5	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR500	ST22	Poultry	31	6	-	optrA_2	poxtA_1	-	optrA-ORG-18; poxtA
VAR570	ST32	Poultry	83	2	-	optrA_2	poxtA_1	-	optrA-ORG-16; poxtA
VAR529	ST22	Poultry	50	6	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR607	ST22	Bovine	111	5	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR558	ST22	Bovine	78	5	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR603	ST22	Pigs and swines	107	5	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR571	ST22	Pigs and swines	84	6	-	optrA_1	poxtA_1	-	optrA only; poxtA
VAR609	ST22	Pigs and swines	113	1	-	optrA_1	poxtA_1	-	optrA only; poxtA

Figure 2. Phylogenetic tree containing the *E. faecium* sequenced samples from this study. MST based on cgMLST results. Branch lengths represent the number of allele differences. Dotted lines are used to help the reader. Imperfect matches for the detected AMR alleles are indicated with an asterisk. A total of 1380 loci after filtering of the allele matrix were used to construct the phylogeny. Dashes for locations indicate that this information was not available. Dashes for genes indicate that no hits were found. Loc., location; nb., number; Gen. org., genetic organization. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

phylogenetic differences were also observed (e.g. *E. faecalis* location 41 or *E. faecium* location 78). For each species, the ST containing the most isolates from this study is discussed in detail below.

E. faecalis—ST480 cluster

Thirteen isolates from this study and 13 datasets obtained from NCBI were assigned to ST480 and were included in this cluster. Except for isolate VAR657, all isolates from this study assigned to ST480 clustered within 20 allele differences on a total of 1945 cgMLST loci after filtering (Figure 4). This group contained isolates from pigs, cattle, dogs and humans collected in various countries between 2013 and 2019 (Table S3). Isolate VAR544 collected from a bovine host showed similarity to isolates collected from humans, since it differed by five alleles compared with samples GCF_014325425.1 and GCF_014325545.1 and eight alleles compared with isolate VAR308 (Figure 4). Except for isolates VAR574 and VAR576, all isolates sequenced in this study were obtained at different locations in Belgium, spread out over 4/6 geographical clusters. *optrA* was detected in all isolates except for three that had a relatively large distance to the rest of the isolates. Despite the relatively small distances, six different alleles of *optrA* were detected in total. For only two of the NCBI isolates, suitable WGS data for constructing a SNP phylogeny were available, but the overall topology in the SNP phylogeny was consistent with results obtained using cgMLST (Figure S4). The SNP address identified a group of nine closely related samples from this study that differed between 10 and 25 SNPs from each other (i.e. SNP address starting with 2.2.2.2), indicating a close phylogenomic relatedness and suggesting a potential epidemiological link, and four additional samples that differed between 50 and 250 SNPs from this group.

E. faecium—ST22 cluster

Seven isolates from this study and 24 datasets obtained from NCBI were assigned to ST22 and were included in this cluster. Six out of seven isolates from this study in this group clustered within 10

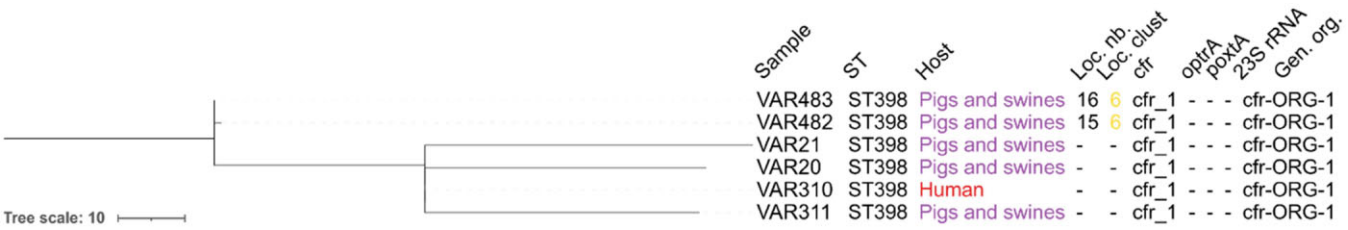


Figure 3. Phylogenetic tree containing the *S. aureus* sequenced samples from this study. MST based on cgMLST results. Branch lengths represent the number of allele differences. Dotted lines are used to help the reader. A total of 2046 loci after filtering of the allele matrix were used to construct the phylogeny. Dashes for locations indicate that this information was not available. Dashes for genes indicate that no hits were found. Loc., location; nb., number; Gen. org., genetic organization. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

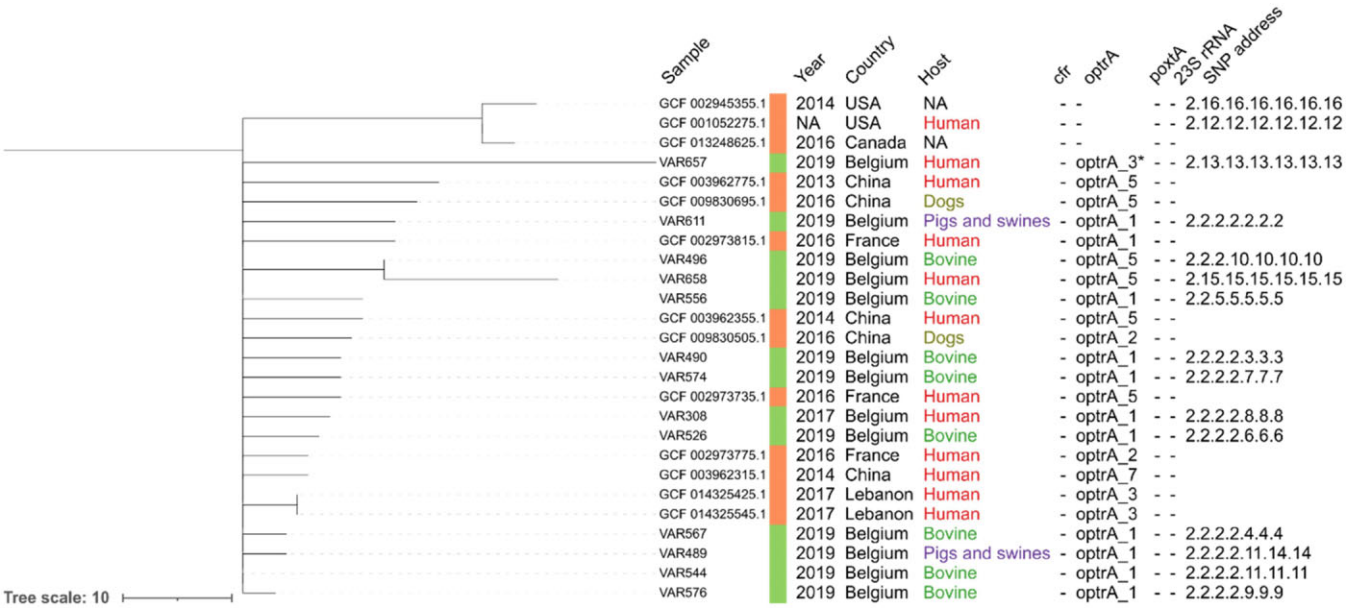


Figure 4. Phylogenetic tree for *E. faecalis* cluster ST480. MST based on cgMLST results. Branch lengths represent the number of allele differences. Dotted lines are used to help the reader. A total of 1945 loci after filtering of the allele matrix were used to construct the phylogeny. Imperfect matches for the detected AMR alleles are indicated with an asterisk. A dash indicates that no hits were found. The colouring represents data retrieved from NCBI (orange) and sequenced in this study (green). Samples without an SNP address did not have suitable Illumina WGS data available (see the Materials and methods section). NA, not available. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

allele differences of each other on a total of 1404 cgMLST loci after filtering (Figure 5). Despite these small distances, the isolates were obtained from various host species. Isolate GCF_008079215.1 (obtained from a dried meat product in Tunisia in 2017)[34] was most closely related to the aforementioned six isolates. Interestingly, this isolate (like other isolates of this ST obtained from NCBI) did not contain either *optrA* or *poxtA* that were present in all isolates from this study for this ST. An SNP-based tree is presented in Figure S5. The SNP address showed differences ranging from 10 to 25 SNPs between the six closely related isolates from this study (i.e. SNP address starting with 4.4.4.4), indicating relatively close phylogenomic relationships.

S. aureus—ST398 cluster

The six isolates from this study were complemented with 899 isolates retrieved from NCBI assigned to ST398. The cgMLST phylogeny for all samples is represented in Figure S6. A subset of 34 samples selected based on their similarity to the samples of this study ($\leq$20 allele differences) is presented in Figure S7. Interestingly, all isolates from this study carried *cfr*, whereas it was absent in all isolates obtained from NCBI in this subset. With the exception of isolate GCF_000638855.1, isolates in the subset were collected from the Netherlands ($n=18$) or Germany ($n=12$), which both share a border with Belgium. The majority of isolates in this subset, including VAR310, were obtained from humans ($n=14$), but various other host species were also observed, including pigs and swine ($n=5$), poultry ($n=4$), horses ($n=4$) and cattle ($n=2$). The coverage of sample VAR20 was not sufficiently high to determine an SNP address, but, for the other isolates from this study, the SNP addresses indicated differences between 50 and 250 SNPs from each other and the isolates retrieved from NCBI, suggesting a relatively distant phylogenomic relationship (Figure S8).

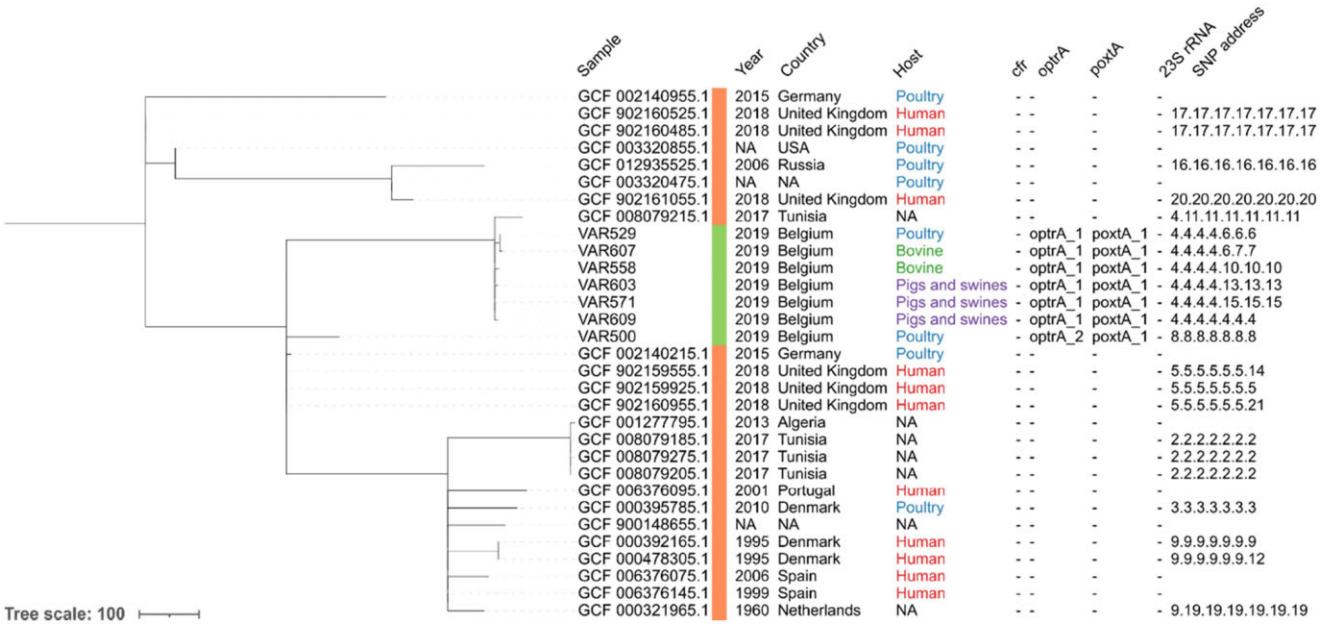


Figure 5. Phylogenetic tree for *E. faecium* cluster ST22. MST based on cgMLST results. Branch lengths represent the number of allele differences. Dotted lines are used to help the reader. A total of 1404 loci after filtering of the allele matrix were used to construct the phylogeny. The colouring represents data retrieved from NCBI (orange) and sequenced in this study (green). A dash indicates that no hits were found. Samples without an SNP address did not have suitable Illumina WGS data available (see the Materials and methods section). NA, not available. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Discussion

This study showed that the LR rate differed widely between different animal host species, with the highest rates occurring in faecal samples from veal calves (16.4%) and nasal samples from sows and pigs (20.5% and 25.7%, respectively). These results indicate that the addition of nasal sampling for enterococci monitoring in food-producing animals could be of added value. Our selective approach combined with a 48 h incubation revealed a much broader reservoir of LR bacteria in food-producing animals than observable through the 2019 OM.[12] In enterococci, LR was mainly conferred by *optrA, poxtA* and *cfr* (in this order), while all LR staphylococci harboured *cfr* with the same genetic organization. Mutations conferring LR were observed less frequently. Co-occurrences of two LR genes in one isolate were reported previously,[8,35] but, to the best of our knowledge, this is the first report of an isolate containing *optrA, poxtA* and *cfr* (VAR473). These results were observed even though the use of linezolid in food-producing animals is not approved.[13] *cfr, optrA* and *poxtA* provide cross-resistance to other antibiotics, with phenicols being the unique common cross-resistance among these three genes.[3–5] Moreover, LR genes were often found to be co-located with phenicol resistance genes (*fexA* or *fexB*). Consequently, phenicol use could also result in co-selection of LR genes. This is important to highlight because florfenicol use has increased each year between 2011 and 2018 in veterinary medicine in Belgium[36] and phenicols have traditionally been considered as one of the antibacterial classes with the lowest importance for human medicine in terms of resistance selection and transfer.[36]

Our study highlighted that a 48 h incubation of the BMD plates enhanced the phenotypic detection of isolates carrying LR determinants compared with a 24 h incubation (147 versus 118, respectively). Similarly, a higher rate of isolates was above the chloramphenicol thresholds after a 48 h incubation. This could suggest that *optrA* and *poxtA* are potentially inducible, similarly to *cfr*,[6] but more experiments are required to confirm this hypothesis. Consequently, we would suggest, for future studies, an additional 24 h incubation period, at least for isolates exhibiting a linezolid MIC of 4 mg/L after 24 h of incubation. Such an extended period of incubation was also recommended by Dejoies *et al.*[15]

Phylogenomic analysis showed that, for both *E. faecalis* and *E. faecium*, isolates belonged to many different STs (28 and 32, respectively), including 11 not previously described, indicating a substantial diversity among LR isolates. Some of the observed STs have also been reported previously in clinical settings, such as *E. faecalis* ST480 and *E. faecium* ST22 (Table S3).[37,38] Overall, large phylogenomic distances were observed for both species between clades of more related isolates (Figures 1 and 2). These observations indicate that reported LR enterococci likely constitute merely the 'tip of the iceberg' and a largely uncharacterized reservoir of LR isolates is circulating. For *S. aureus*, all six isolates were classified as ST398 with relatively large SNP distances, however, between most isolates. Although too few Belgian staphylococci isolates were included to make definitive claims, this suggests a similar trend as observed for *Enterococcus* spp.

Locations that were sampled multiple times typically contained mostly closely related isolates differing by only a limited number of SNPs, suggesting a potential clonal relationship, which would therefore be prime candidates for more detailed epidemiological investigation. Nevertheless, for several locations, isolates with phylogenetically distant strains, or even different species (e.g. location 34), were observed. The presence of multiple unrelated LR

isolates at a single location could indicate that even more variation exists at the sampled locations than observed here. On the other hand, clusters of closely related isolates were not limited to a single location or geographical cluster, but were often spread out across the whole country, indicating transmission of strains within Belgium.

While certain lineages are commonly associated with hospital outbreaks (i.e. ST6 for *E. faecalis*[39] and ST180, ST117 or ST78 for *E. faecium*;[40] Table S3), sharing of *E. faecalis* or *E. faecium* strains between livestock and humans has also been reported.[41,42] In this study, we observed *E. faecalis* isolates with high genomic similarity collected from various host species, including humans (e.g. Figure 4), suggesting that a spillover of such strains could occur. Additionally, since enterococci in livestock can serve as reservoirs for resistance genes,[41,43] these strains pose a substantial threat for AMR transmission to humans, even when direct transmission is unlikely.

In general, no clear correlation was observed between phylogenies and AMR genotypes, for all three species. These findings suggest that strains can easily exchange or acquire genetic material to obtain LR genes. In some cases, genotypic LR profiles were not consistent between isolates obtained at the same sampling location, indicating a non-unique source of LR at a single place.

In conclusion, this study showed that LR is present in a wide variety of isolates circulating in the agricultural sector in Belgium. Related isolates were recovered from different host species, including humans. Geographically, related isolates were spread out across the whole country and even across international borders. The lack of correlation between the phylogenies and the LR genes suggests that these genomic features are easily transferable as was already shown in previous studies.[44–46] Importantly, a substantial amount of genomic diversity was observed, including the detection of several features that were not yet documented, such as potential novel variants of resistance genes, new genetic organizations and new STs, indicating that much diversity exists among enterococci circulating in the agricultural sector, which remains hidden when only considering isolates obtained from human patients. This study showed that LR strains, genes and mutations are spread across the whole country and internationally, posing a risk to human health. The cross-selection of LR through the use of antibiotics currently listed as of lowest importance for human medicine (among others phenicols) should be considered in future action plans against AMR and in future objectives for rational antibiotic use. LR dedicated monitoring based on WGS analysis should be considered to monitor LR and prevent potential outbreaks. Similar studies in other countries, as recently conducted in Italy,[47] would increase knowledge and awareness about LR in the agricultural sector in a One Health perspective.

Acknowledgements

Parts of this work were presented at the 2019 and 2020 annual scientific meetings of the One Health European Joint Programme in the form of posters (posters 56 and 147, respectively).

We thank the Federal Agency for the Safety of the Food Chain for collecting the samples used in this study and providing the metadata. Technical support from A. Radu and D. Petrone was highly appreciated. We also thank Michèle Driesen and Xavier Simons from the Veterinary Epidemiology Service at Sciensano for the geographical coordinates of the samples. We thank the technicians of the Transversal Activities in Applied Genomics Service at Sciensano (Belgium) for performing the next-generation sequencing runs. We also thank the team of 'Hygiène Hospitalière' from St Luc Hospital, Woluwe-Saint-Lambert, Belgium.

Funding

This work was supported by funding from the European Union's Horizon 2020 Research and Innovation programme under Grant Agreement No. 773830: One Health European Joint Programme, and from Sciensano.

Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 and Figures S1 to S8 are available as Supplementary data at *JAC* Online.

References

1 WHO. Critically Important Antimicrobials for Human Medicine 6th Revision 2018. 2019. https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1.

2 Prystowsky J, Siddiqui F, Chosay J *et al.* Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. *Antimicrob Agents Chemother* 2001; **45**: 2154–6.

3 Long KS, Poehlsgaard J, Kehrenberg C *et al.* The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. *Antimicrob Agents Chemother* 2006; **50**: 2500–5.

4 Wang Y, Lv Y, Cai J *et al.* A novel gene, *optrA*, that confers transferable resistance to oxazolidinones and phenicols and its presence in *Enterococcus faecalis* and *Enterococcus faecium* of human and animal origin. *J Antimicrob Chemother* 2015; **70**: 2182–90.

5 Antonelli A, D'Andrea MM, Brenciani A *et al.* Characterization of *poxtA*, a novel phenicol-oxazolidinone-tetracycline resistance gene from an MRSA of clinical origin. *J Antimicrob Chemother* 2018; **73**: 1763–9.

6 Schwarz S, Werckenthin C, Kehrenberg C. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. *Antimicrob Agents Chemother* 2000; **44**: 2530–3.

7 Sharkey LKR, Edwards TA, O'Neill AJ. ABC-F proteins mediate antibiotic resistance through ribosomal protection. *mBio* 2016; **7**: e01975.

8 Li D, Wang Y, Schwarz S *et al.* Co-location of the oxazolidinone resistance genes optrA and cfr on a multiresistance plasmid from *Staphylococcus sciuri*. *J Antimicrob Chemother* 2016; **71**: 1474–8.

9 Paridaens H, Coussement J, Argudín MA *et al.* Clinical case of *cfr*-positive MRSA CC398 in Belgium. *Eur J Clin Microbiol Infect Dis* 2017; **36**: 1527–9.

10 Argudín MA, Youzaga S, Dodémont M *et al.* Detection of *optrA*-positive enterococci clinical isolates in Belgium. *Eur J Clin Microbiol Infect Dis* 2019; **38**: 985–7.

11 European Food Safety Authority, ECDC. EU summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. *EFSA J* 2015; **13**: 4036.

12 Federal Agency for the Safety of the Food Chain. 2020. http://www.favv.be/productionanimale/antibioresistance/resultats/#sciensano.

13 European Food Safety Authority. Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal *Escherichia coli* and *Enterococcus* spp. from food animals. *EFSA J* 2008; **141**: 1–44.

14 Werner G, Fleige C, Klare I *et al*. Validating a screening agar for linezolid-resistant enterococci. *BMC Infect Dis* 2019; **19**: 1078.

15 Dejoies L, Boukthir S, Péan de Ponfilly G *et al*. Performance of commercial methods for linezolid susceptibility testing of *Enterococcus faecium* and *Enterococcus faecalis*. *J Antimicrob Chemother* 2020; **75**: 2587–93.

16 EUCAST. Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 10.0. 2020. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf.

17 EUCAST. Epidemiological Cut-Off - MIC EUCAST. 2020. https://mic.eucast.org/search/.

18 Leinonen R, Sugawara H, Shumway M on behalf of the International Nucleotide Sequence Database Collaboration. The sequence read archive. *Nucleic Acids Res* 2011; **39**: D19–21.

19 Bogaerts B, Nouws S, Verhaegen B *et al*. Validation strategy of a bioinformatics whole genome sequencing workflow for Shiga toxin-producing *Escherichia coli* using a reference collection extensively characterized with conventional methods. *Microb Genom* 2021; **7**: mgen000531.

20 Jolley KA, Bliss CM, Bennett JS *et al*. Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. *Microbiology (Reading)* 2012; **158**: 1005–15.

21 Hasman H, Clausen PTLC, Kaya H *et al*. LRE-Finder, a Web tool for detection of the 23S rRNA mutations and the *optrA, cfr, cfr*(B) and *poxtA* genes encoding linezolid resistance in enterococci from whole-genome sequences. *J Antimicrob Chemother* 2019; **74**: 1473–6.

22 Bortolaia V, Kaas RS, Ruppe E *et al*. ResFinder 4.0 for predictions of phenotypes from genotypes. *J Antimicrob Chemother* 2020; **75**: 3491–500.

23 Milne I, Stephen G, Bayer M *et al*. Using Tablet for visual exploration of second-generation sequencing data. *Brief Bioinform* 2013; **14**: 193–202.

24 Bratcher HB, Corton C, Jolley KA *et al*. A gene-by-gene population genomics platform: *de novo* assembly, annotation and genealogical analysis of 108 representative *Neisseria meningitidis* genomes. *BMC Genomics* 2014; **15**: 1138.

25 Neumann B, Prior K, Bender JK *et al*. A core genome multilocus sequence typing scheme for *Enterococcus faecalis*. *J Clin Microbiol* 2019; **57**: e01686–18.

26 de Been M, Pinholt M, Top J *et al*. Core genome multilocus sequence typing scheme for high-resolution typing of *Enterococcus faecium*. *J Clin Microbiol* 2015; **53**: 3788–97.

27 Zhou Z, Alikhan N-F, Sergeant MJ *et al*. GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. *Genome Res* 2018; **28**: 1395–404.

28 Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* 2019; **47**: W256–9.

29 Davis S, Pettengill JB, Luo Y *et al*. CFSAN SNP Pipeline: an automated method for constructing SNP matrices from next-generation sequence data. *PeerJ Comput Sci* 2015; https://doi.org/10.7717/peerj-cs.20.

30 Kumar S, Stecher G, Li M *et al*. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018; **35**: 1547–9.

31 Van Driessche L, Vanneste K, Bogaerts B *et al*. Isolation of drug-resistant *Gallibacterium anatis* from calves with unresponsive bronchopneumonia, Belgium. *Emerg Infect Dis* 2020; **26**: 721–30.

32 Dallman T, Ashton P, Schafer U *et al*. SnapperDB: a database solution for routine sequencing analysis of bacterial isolates. *Bioinformatics* 2018; **34**: 3028–9.

33 Nouws S, Bogaerts B, Verhaegen B *et al*. Impact of DNA extraction on whole genome sequencing analysis for characterization and relatedness of Shiga toxin-producing *Escherichia coli* isolates. *Sci Rep* 2020; **10**: 14649.

34 Zommiti M, Cambronel M, Maillot O *et al*. Evaluation of probiotic properties and safety of *Enterococcus faecium* isolated from artisanal Tunisian meat 'Dried Ossban'. *Front Microbiol* 2018; **9**: 1685.

35 Lazaris A, Coleman DC, Kearns AM *et al*. Novel multiresistance cfr plasmids in linezolid-resistant methicillin-resistant *Staphylococcus epidermidis* and vancomycin-resistant *Enterococcus faecium* (VRE) from a hospital outbreak: co-location of *cfr* and *optrA* in VRE. *J Antimicrob Chemother* 2017; **72**: 3252–7.

36 Belgian Veterinary Surveillance of Antibacterial Consumption, National Consumption Report, 2019. 2020. https://www.fagg.be/sites/default/files/content/belvetsac_sanimed_rapport_2019_finaal.pdf.

37 Egan SA, Shore AC, O'Connell B *et al*. Linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis* from hospitalized patients in Ireland: high prevalence of the MDR genes *optrA* and *poxtA* in isolates with diverse genetic backgrounds. *J Antimicrob Chemother* 2020; **75**: 1704–11.

38 Tedim AP, Ruiz-Garbajosa P, Corander J *et al*. Population biology of intestinal *Enterococcus* isolates from hospitalized and nonhospitalized individuals in different age groups. *Appl Environ Microbiol* 2015; **81**: 1820–31.

39 Leavis HL, Bonten MJM, Willems RJL. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* 2006; **9**: 454–60.

40 Eisenberger D, Tuschak C, Werner M *et al*. Whole-genome analysis of vancomycin-resistant *Enterococcus faecium* causing nosocomial outbreaks suggests the occurrence of few endemic clonal lineages in Bavaria, Germany. *J Antimicrob Chemother* 2020; **75**: 1398–404.

41 Hammerum AM. Enterococci of animal origin and their significance for public health. *Clin Microbiol Infect* 2012; **18**: 619–25.

42 Gouliouris T, Raven KE, Ludden C *et al*. Genomic surveillance of *Enterococcus faecium* reveals limited sharing of strains and resistance genes between livestock and humans in the United Kingdom. *mBio* 2018; **9**: e01780–18.

43 Rolain J-M. Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. *Front Microbiol* 2013; **4**: 173.

44 Ntokou E, Stathopoulos C, Kristo I *et al*. Intensive care unit dissemination of multiple clones of linezolid-resistant *Enterococcus faecalis* and *Enterococcus faecium*. *J Antimicrob Chemother* 2012; **67**: 1819–23.

45 Huang J, Chen L, Wu Z *et al*. Retrospective analysis of genome sequences revealed the wide dissemination of *optrA* in Gram-positive bacteria. *J Antimicrob Chemother* 2017; **72**: 614–6.

46 Freitas AR, Tedim AP, Novais C *et al*. Comparative genomics of global *optrA*-carrying *Enterococcus faecalis* uncovers a common chromosomal hotspot for *optrA* acquisition within a diversity of core and accessory genomes. *Microb Genom* 2020; **6**: e000350.

47 Fioriti S, Morroni G, Coccitto SN *et al*. Detection of oxazolidinone resistance genes and characterization of genetic environments in enterococci of swine origin, Italy. *Microorganisms* 2020; **8**: 2021.