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Detection of transferable oxazolidinone resistance determinants in *Enterococcus faecalis* and *Enterococcus faecium* of swine origin in Sichuan Province, China

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ABSTRACT

Objectives: The aim of this study was to detect transferable oxazolidinone resistance determinants (*cfr*, *optrA* and *poxtA*) in *Enterococcus faecalis* and *Enterococcus faecium* isolates of swine origin in Sichuan Province, China.

Methods: A total of 158 enterococcal isolates (93 *E. faecalis* and 65 *E. faecium*) isolated from 25 large-scale swine farms (2016–2017) were screened for the presence of *cfr*, *optrA* and *poxtA* by PCR. The genetic environments of *cfr*, *optrA* and *poxtA* were characterised by whole-genome sequencing. Transfer of oxazolidinone resistance determinants was determined by conjugation or electrotransformation experiments.

Results: The transferable oxazolidinone resistance determinants *cfr*, *optrA* and *poxtA* were detected in zero, six and one enterococcal isolates, respectively. The *poxtA* gene in one *E. faecalis* isolate was located on a 37 990-bp plasmid that co-harboured *fexB*, *cat*, *tet*(L) and *tet*(M) and could be conjugated to *E. faecalis* JH2-2. One *E. faecalis* isolate harboured two different OptrA variants, including one variant with a single substitution (Q219H) that has not been reported previously. Two *optrA*-carrying plasmids, pC25-1 (45 581 bp) and pC54 (64 500 bp), shared a 40 494-bp identical region containing the genetic context IS*1216E*–*fexA*–*optrA*–*erm*(A)–IS*1216E* that could be electrotransformed into *Staphylococcus aureus*. Four different chromosomal *optrA* gene clusters were found in five strains, in which *optrA* was associated with Tn*554* or Tn*558* inserted into the *radC* gene.

Conclusion: This study highlights the fact that mobile genetic elements, such as plasmids, IS*1216E*, Tn*554* and Tn*558*, may facilitate the horizontal transmission of *optrA* and *poxtA* genes.

1. Introduction

Enterococci are ubiquitous Gram-positive bacteria widely distributed in the natural environment and gastrointestinal tract of humans and animals [1,2]. Enterococci, mostly *Enterococcus faecalis* and *Enterococcus faecium*, have recently been identified as common causes of hospital-associated infections [3]. Oxazolidinones (linezolid and tedizolid) are effective antimicrobial agents for treating infections caused by multidrug-resistant Gram-positive bacteria, including vancomycin-resistant enterococci (VRE) [4,5]. Linezolid was the first commercially available oxazolidinone, which can inhibit protein synthesis by binding to the peptidyl transferase centre of the bacterial 23S rRNA [6]. During the past few decades, the occurrence of linezolid-resistant *E. faecalis* and *E. faecium* has increased in hospitals worldwide [3], presenting a great challenge for the treatment of VRE.

Ribosomal mutations, especially the G2576T (*Escherichia coli* numbering) mutation in the 23S rRNA gene [6], are the predominant mechanism of oxazolidinone resistance in enterococci. The transferable oxazolidinone resistance determinant *cfr* has been reported in enterococci in several regions worldwide [7]. Recently, another transferable oxazolidinone resistance gene (*optrA*) was reported in *E. faecium* and *E. faecalis* isolated both from human and food-producing animals in China in 2015 [8]. The *optrA* gene encodes an ABC-F protein that can protect the bacterial

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ribosome from antibiotic inhibition and confers cross-resistance to phenicols (chloramphenicol and florfenicol) and oxazolidinones [8,9]. The occurrence of *optrA* has not only been reported in Asia [10–14] but also in other geographical areas including Europe, South America and North America [3,15–20]. The phenicol–oxazolidinone–tetracycline resistance gene *poxtA* encodes a ribosomal protection protein of the ABC-F family showing 32% identical to OptrA. The *poxtA* gene was first reported in a clinical methicillin-resistant *Staphylococcus aureus* (MRSA) isolate in 2018 [21] and has also been found in *E. faecium* and *E. faecalis* [22,23].

China's swine industry is prominent in Sichuan Province. Although oxazolidinones are not used in the swine industry, florfenicol is widely used for the treatment of bacterial infections in swine, which may promote the dissemination of *cfr*, *optrA* and *poxtA* genes on swine farms. The aim of this study was to detect the transferable oxazolidinone resistance determinants *cfr*, *optrA* and *poxtA* in *E. faecalis* and *E. faecium* of swine origin in Sichuan Province, China.

2. Materials and methods

2.1. Bacterial strains, detection of cfr, optrA and poxtA, and antimicrobial susceptibility testing

A total of 250 faecal swabs were collected from healthy swine on 25 large-scale swine farms between June 2016 and May 2017 in Sichuan Province. Ten faecal swabs were randomly collected from each farm. Enterococcal isolates were identified using an automated system (BD Diagnostic Systems, Sparks, MD, USA). All strains were screened for the presence of *cfr*, *optrA* and *poxtA* by PCR as described previously [8,23]. Positive PCR products were sequenced by Chengdu Sangon Biological Engineering Technology & Services Co, Ltd. (Chengdu, China). Antimicrobial susceptibility testing to linezolid, chloramphenicol, florfenicol, erythromycin, doxycycline, ampicillin, fosfomycin and vancomycin was performed by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [24]. *Enterococcus faecalis* ATCC 29212 was used as a quality control strain in minimum inhibitory concentration (MIC) determinations.

2.2. Whole-genome sequencing (WGS) and analysis

Genomic DNA of *optrA*- and *poxtA*-positive strains was extracted using a MiniBEST Bacteria Genomic DNA Extraction Kit (TaKaRa, Dalian, China). WGS was performed on an Illumina HiSeq platform (Illumina Inc., San Diego, CA, USA) with 400-bp paired-end reads and 200-fold average coverage. Draft genomes were assembled using SPAdes_3.12.0. The whole genomes of strains C25 and C54 that contained plasmids harbouring *optrA* were further sequenced using a PacBio RS II sequencing instrument (Pacific Biosciences, Menlo Park, CA, USA) with 100-fold average read depth. The chromosomes and plasmids were respectively assembled into one scaffold using the software SMRT® Portal v.3.2.0 (Pacific Biosciences). Sequence types (STs) and acquired antimicrobial resistance genes were identified using MLST 2.0 (multilocus sequence typing) (https://cge.cbs.dtu.dk/services/MLST/) and ResFinder 3.1 (https://cge.cbs.dtu.dk/services/ResFinder/), respectively. The genetic environments of *optrA* and *poxtA* were analysed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Transfer experiments

Conjugation experiments were performed by filter mating using rifampicin-resistant *E. faecalis* JH2-2 as the recipient strain with selection on brain–heart infusion agar plates containing 25 mg/L rifampicin and 2 mg/L linezolid. Electrotransformation experiments were performed using *S. aureus* RN4220 as the recipient and 2 mg/L linezolid as a selection marker. Transconjugants or transformants were further investigated through *optrA* and *poxtA* detection and antimicrobial susceptibility testing.

2.4. Nucleotide sequence accession numbers

The *optrA* and *poxtA* gene cluster sequences of all distinct isolates as well as the genomes of strains C25 and C54 characterised in this study have been deposited in GenBank and assigned accession nos. **MK251150**–**MK251154** (chromosomal *optrA* gene clusters), **MK861852** (pC10), **CP030042** (C25 chromosome), **CP030043** (pC25-1), **CP030044** (pC25-2), **CP030045** (C54 chromosome) and **CP030046** (pC54).

3. Results and discussion

A total of 158 enterococci strains (93 *E. faecalis* and 65 *E. faecium*) were isolated from 25 large-scale swine farms in Sichuan Province. The transferable oxazolidinone resistance determinants *cfr*, *optrA* and *poxtA* were detected in zero, six and one enterococcal strains, respectively (Table 1). The detection rate of *optrA* in this study (6/158; 3.8%) is lower than that reported by Wang et al. [8] who found that 24.8% (37/149) enterococci strains of swine origin harboured *optrA*. The different detection rates of *optrA* may be due to the fact that they were isolated in different provinces as well as the use of different antimicrobial agents. The seven *optrA*- or

Table 1
Antimicrobial resistance phenotypes and acquired antimicrobial resistance genes in *optrA*-and *poxtA*-positive enterococcal strains.

Strain	MLST	MIC (mg/L)					Acquired antimicrobial resistance determinants				
		LNZ	CHL	FFC	ERY	DOX	Oxazolidinone/MLS_B	Aminoglycoside	Phenicol	Tetracycline	Trimethoprim
E. faecalis C25	ST691	16	32	32	64	4	*optrA*, *erm*(A), *lsa*(A)	*spc*	*fexA*	–	–
E. faecalis C54	ST74	8	32	32	64	2	*optrA*, *erm*(A), *lsa*(A)	–	*fexA*	–	–
E. faecium GJA5	ST1051	4	64	128	256	32	*optrA*, *msr*(C), *lnu*(B), *erm*(A), *erm*(B)	*aadE*, *spc*, *aacA–aphD*, *aphA-3*	*fexB*, *cat*	*tet*(L), *tet*(M)	–
E. faecium SC1	ST1011	2	64	128	128	64	*optrA*, Δ*erm*(A), *erm*(T), *lnu*(B), *erm*(B)	*aphA-3*	*fexA*, *fexB*, *cat*	*tet*(L), *tet*(M)	*dfrG*
E. faecium SC18	ST1146	4	64	128	128	32	*optrA*, *erm*(A), *erm*(B)	*aphA-3*, *aadE*	*fexA*, *fexB*, *cat*	*tet*(L), *tet*(M)	*dfrG*
E. faecium YG1	ST486	4	128	128	128	32	*optrA*, *erm*(A), *erm*(B), *lnu*(B)	*aphA-3*, *aacA–aphD*	*fexA*, *fexB*	*tet*(L), *tet*(M)	*dfrG*
E. faecalis C10	ST404	4	64	128	64	32	*poxtA*, *erm*(B), *lnu*(B)	*aphA-3*, *aacA–aphD*	*fexB*, *cat*	*tet*(L), *tet*(M)	

MLST, multilocus sequence typing; MIC, minimum inhibitory concentration; LNZ, linezolid; CHL, chloramphenicol; FFC, florfenicol; ERY, erythromycin; DOX, doxycycline; MLS_B, macrolide–lincosamide–streptogramin B.

Table 2
Genetic environment of *optrA* variants in *Enterococcus faecalis* and *Enterococcus faecium* strains characterised in this study.

Strain	OptrA				Co-located antimicrobial resistance genes
	Variant	Alterations	Location (kb)	Array[a]	
E. faecalis C25	RDK	I104R, Y176D, E256K	Plasmid (45 kb)	a	*fexA*, *erm*(A)
	H	Q219H	Chromosome	c	*fexA*, *erm*(A)
E. faecalis C54	RDK	I104R, Y176D, E256K	Plasmid (64 kb)	b	*fexA*, *erm*(A)
E. faecium GJA5	EDD	K3E, Y176D, G393D	Chromosome	d	*erm*(A)
E. faecium SC1	EDM	K3E, Y176D, I622M	Chromosome	e	*fexA*, Δ*erm*(A)
E. faecium SC18	DDD	G40D, Y176D, G393D	Chromosome	f	*fexA*, *erm*(A)
E. faecium YG1	DDD	G40D, Y176D, G393D	Chromosome	f	*fexA*, *erm*(A)

[a] Gene array as depicted in Fig. 3.

poxtA-carrying strains isolated in the current study showed decreased susceptibility to linezolid, with MICs ranging from 2–16 mg/L. All of the strains were resistant to chloramphenicol (MICs of 32–128 mg/L), florfenicol (MICs of 32–128 mg/L) and erythromycin (MICs of 64–256 mg/L) but were susceptible to ampicillin (MIC ≤ 2 mg/L), fosfomycin (MIC ≤ 4 mg/L) and vancomycin (MIC ≤ 2 mg/L). The four *optrA*-positive *E. faecium* isolates also showed resistance to doxycycline (MICs of 32–64 mg/L). The acquired antimicrobial resistance genes are shown in Table 1. Six strains harboured the oxazolidinone–phenicol resistance gene *optrA*, the phenicol resistance genes *fexA* and/or *fexB*, and the erythromycin resistance genes *erm*(A) and/or *erm*(B). It is noteworthy that *E. faecalis* strain C25 harboured two copies of *optrA*. The tetracycline resistance genes *tet*(L) and *tet*(M) were also present in one *poxtA*-positive *E. faecalis* and four *optrA*-positive *E. faecium* isolates. The *optrA*-positive enterococci strains belonged to six different STs (Table 1), indicating the genetic diversity of the *optrA*-positive enterococci.

Alignment of the deduced six OptrA amino acid sequences revealed that the six enterococci strains harboured five different OptrA variants compared with that found in *E. faecalis* E349 [8] (Table 2). Four of these variants have been reported previously [25]. It is worth noting that *E. faecalis* strain C25 harboured two different *optrA* variants, including one variant with a single substitution (Q219H) that has not been reported previously. Different linezolid MICs were also present in the six enterococci strains harbouring different *optrA* variants (Table 1). Until now, many *optrA* variants have been reported in *Enterococcus* and *Staphylococcus* that exhibit varied linezolid MICs. Cai et al. speculated that *optrA* mutations, the genetic context of *optrA*, bacterial host and other factors might regulate the expression of *optrA*, resulting in different levels of linezolid resistance [25]. More studies should be carried out to further characterise this phenomenon.

The genetic environments of *poxtA* and *optrA* were characterised by WGS. *Enterococcus faecalis* strain C10 harboured a *poxtA*-carrying plasmid named pC10. Plasmid pC10 was 37 990 bp in length with a GC content of 34.83%. It encodes 46 open-reading frames (ORFs) and could be conjugated to *E. faecalis* JH2-2. Plasmid pC10 harbours the replication initiator gene *repA* belonging to the *rep2* family [26]. Other resistance genes, including *fexB*, *tet*(M), *tet*(L) and *cat*, were also found in pC10. BLAST analysis showed that pC10 was 99.91% identical to the corresponding region of plasmid pLS170308 (GenBank accession no. **CP025078**) with 55% coverage (Fig. 1). pLS170308 was found in *E. faecium* of *Moschus berezovskii* origin in Sichuan Province, indicating that pC10 and pLS170308 may have the same origin. The *poxtA* gene in plasmid pC10 was flanked by IS*1216E* (Fig. 1), similar to MSRA strain AOUC-0915 [21].

Enterococcus faecalis strain C25 harboured a chromosomal *optrA* gene cluster and an *optrA*-carrying plasmid designated pC25-1, and this strain also carried another plasmid (pC25-2) that was not associated with antimicrobial resistance genes. pC25-1 is 45 581 bp in length with a GC content of 30.73% and encodes 59 ORFs (Fig. 2a). The replication initiator gene *repA* of pC25-1 belongs to the *rep9* family [26]. Some genes, such as conjugal transfer gene *traB*, type III secretion system gene *prgN* and partitioning gene *parA*, were found in pC25-1 and may be associated with plasmid transfer and stability. BLAST analysis showed that pC25-1 had 99.9% nucleotide identity with *E. faecalis* plasmid pEF123 (GenBank accession no. **KX579977**) with 58% coverage. *Enterococcus faecalis* strain C54 contained an *optrA*-carrying plasmid (pC54) that was 64 500 bp in length with a GC content of 31.53% and encoding 79 ORFs (Fig. 2a). The replication gene *repA* (1011 bp) in both pC25-1 and pC54 showed 98.8% nucleotide identities with pTEF2 from *E. faecalis* V583 (**AE016831**). pC25-1 and pC54 also shared a 40 494-bp identical region that contained the genetic contexts IS*1216E*–*fexA*–*optrA*–*erm*(A)–IS*1216E* and IS*1216E*–*bcrB*–*bcrD*–*bcrA*–*bcrR*–IS*1216E* (Fig. 2b), indicating that the two plasmids had the same origin. A similar genetic context IS*1216E*–*fexA*–*optrA*–*erm*(A)–IS*1216E* has been found in many diverse *optrA*-carrying plasmids [25], indicating that IS*1216E* might promote the co-transfer of *optrA*, *fexA* and *erm*(A) among different plasmids. Although conjugation experiments for all six *optrA*-positive enterococci strains failed, the *optrA*-carrying plasmids pC25-1 and pC54 could

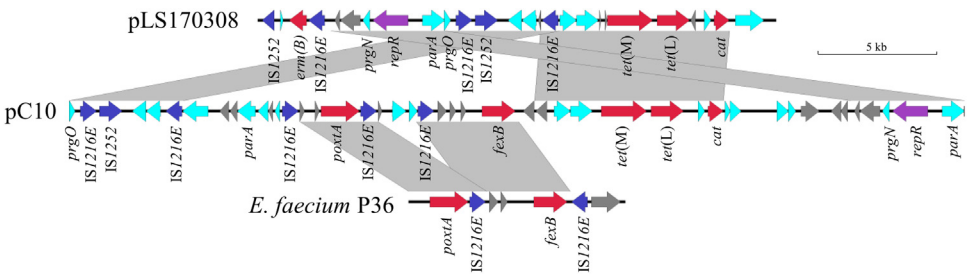

Fig. 1. Schematic representation of *poxtA*-harbouring plasmid pC10. Structures are drawn to scale from GenBank accession nos. **MK861852** (pC10), **KP834591** (*Enterococcus faecium* P36) and **CP025078** (pLS170308). Genes and open-reading frames (ORFs) are shown as arrows, and their orientations of transcription are indicated by the arrowheads. Shared regions of >99% nucleotide sequence identity are indicated by grey shading. Antimicrobial resistance genes are in red, insertion sequences (ISs) are in navy blue, hypothetical protein genes are in grey, replication genes are in purple and other protein genes are in light blue.

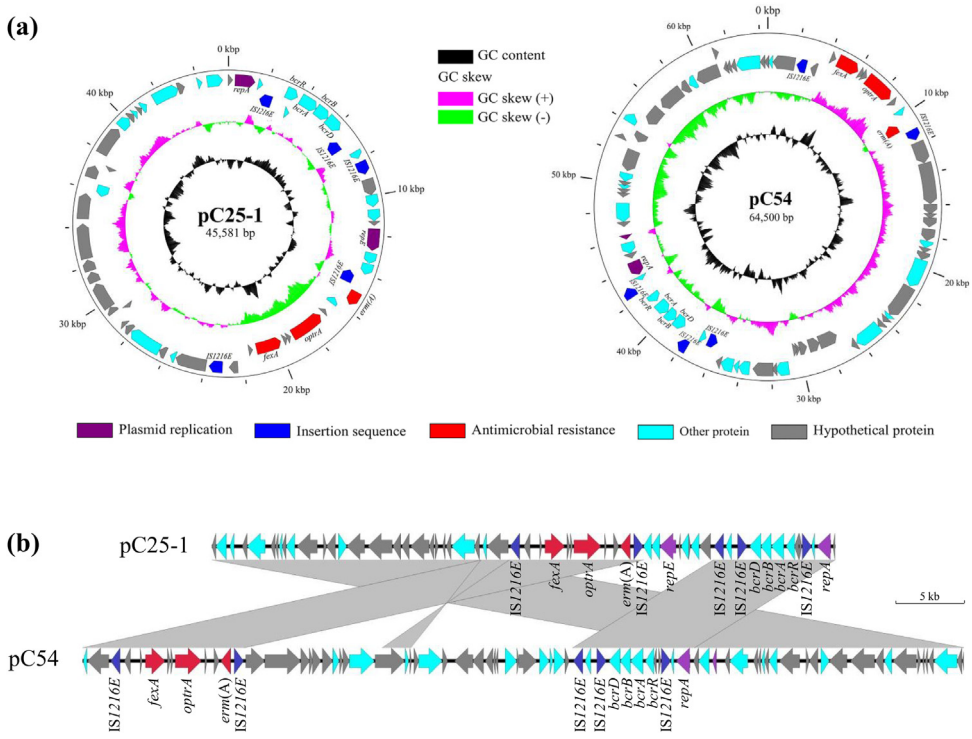

Fig. 2. (a) Genetic map of *optrA*-harbouring plasmids pC25-1 and pC54 and (b) schematic representation of plasmid pC25-1 in comparison with pC54. The elements are colour-coded after their predicted functions, and positions of predicted coding sequences transcribed in the clockwise orientation. Shared regions with >99% identity are indicated by grey shading.

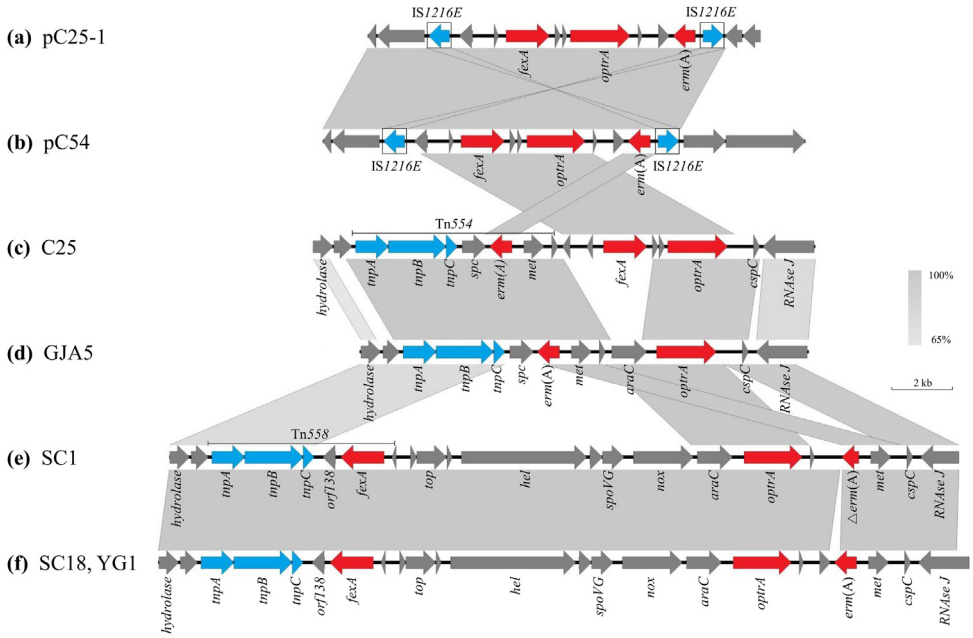

Fig. 3. Genetic environments of *optrA* in six *optrA*-carrying enterococcal isolates. Antimicrobial resistance genes are in red, transposase or integrase genes are in blue and other genes are in grey. Grey shaded areas represent regions of >65% nucleotide sequence identity. Arrows indicate the positions and orientations of the genes.

be electrotransformed into *S. aureus* RN4220 and confer resistance to linezolid (MIC = 8 mg/L), chloramphenicol (MIC = 64 mg/L), florfenicol (MIC = 64 mg/L) and erythromycin (MIC = 128 mg/L).

Four different chromosomal *optrA* gene clusters were found in five strains (Fig. 3c–f), in which *optrA* was associated with Tn*554* or Tn*558* inserted into the *radC* gene [27,28]. Transposon Tn*554* was detected upstream of *fexA*–*optrA*(H) segment in *E. faecalis* C25 (Fig. 3c) and of *araC*–*optrA*(EDD) in *E. faecium* GJA5 (Fig. 3d). The *optrA*(H) flanking region in *E. faecalis* C25 (ST691) showed 99% identity to the corresponding region in *E. faecalis* A101 that was isolated from a faecal sample of a healthy Chinese person [25]. Transposon Tn*558*, as well as four ORFs encoding topoisomerase, helicase, septation protein SpoVG and putative NADH oxidase, respectively, was found upstream of the *araC*–*optrA*(EDM) segment in *E. faecium* SC1 (Fig. 3e). Apart from Tn*554* containing truncated *erm*(A), a *met* segment was observed downstream of the

araC–optrA(EDM) segment. The chromosomal *optrA* gene cluster in *E. faecium* SC1 shared 99% identity with that in *E. faecalis* strain E016 of Chinese human origin [29]. *Enterococcus faecium* SC18 (ST1146) and YG1 (ST486) shared an identical chromosomal *optrA* (DDD) gene cluster that contained a complete copy of *erm*(A) compared with the corresponding region in *E. faecium* SC1 (Fig. 3e,f).

In conclusion, the present study detected transferable oxazolidinone resistance determinants in *E. faecalis* and *E. faecium* of swine origin in Sichuan Province, China. Five different *optrA* variants associated with distinct genetic contexts were characterised by WGS. Mobile genetic elements, such as plasmids, IS*1216E*, Tn*554* and Tn*558*, may facilitate the horizontal transmission of *optrA* and *poxtA*. The presence of other antimicrobial resistance genes, including *fexA*, *fexB* and *erm*(A), may lead to the co-selection of *optrA* and *poxtA*. More attention should be paid to the dissemination of transferable oxazolidinone resistance determinants in Gram-positive bacteria of food-producing animal origin, since animal farms might serve as reservoirs of transferable oxazolidinone resistance determinants that could spread to humans through the food chain.

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Competing interests

None declared.

Ethical approval

Not required.

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