Detection of the staphylococcal multiresistance gene \textit{cfr} in \textit{Escherichia coli} of domestic-animal origin

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**Objectives:** To investigate the presence and the genetic environment of the multiresistance gene \textit{cfr} in \textit{Escherichia coli} found in domestic animals.

**Methods:** A total of 1230 \textit{E. coli} isolates, collected from pigs, chickens and ducks, were screened by PCR for the \textit{cfr} gene. The location of the \textit{cfr} gene was determined by Southern blotting, the transferability of \textit{cfr} gene was tested by conjugation and transformation, and the regions flanking the \textit{cfr} gene were sequenced by a modified random primer walking strategy. The location of the \textit{cfr} promoter sequence was analysed by mapping the \textit{cfr} transcription start site using rapid amplification of 5'-cDNA ends (5'-RACE).

**Results:** Only a single strain from the nasal swab of a pig harboured the \textit{cfr} gene. Southern blotting indicated that the \textit{cfr} gene was located on a \~{}110 kb plasmid, designated pEC-01. A \textit{cfr}-carrying segment of 1545 bp with a sequence identical to that of the \textit{cfr}-harbouring plasmid pSCFS1 was flanked by two IS26 elements in the same orientation. The IS26 transposition created a new hybrid promoter in which the \textit{cfr} promoter sequence was analysed by mapping the \textit{cfr} promoter sequence.

**Conclusions:** To the best of our knowledge, this is the first report of the \textit{cfr} gene in a naturally occurring \textit{E. coli} strain. Continued surveillance of the presence of the \textit{cfr} gene in Gram-negative bacteria of domestic-animal origin is warranted.

**Keywords:** linezolid resistance, inter-genus transfer, food safety

**Introduction**

The staphylococcal multiresistance gene \textit{cfr} mediates resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, streptogramin A and the 16-membered macrolides spiramycin and josamycin.\textsuperscript{1,2} Each of these antimicrobial classes is used in the treatment of staphylococcal infections in both human and veterinary medicine. The \textit{cfr} gene was originally identified in coagulase-negative staphylococci (CoNS) from animals, but has since been found in a limited number of \textit{Staphylococcus aureus} isolates and CoNS from humans and animals.\textsuperscript{3–6} Recently, this gene has also been sporadically identified in other Gram-positive bacteria, such as \textit{Bacillus} spp. and \textit{Enterococcus faecalis}.\textsuperscript{7,8} Plasmids seem to play an important role in the inter-species and inter-genus transfer of this multiresistance gene. Under laboratory conditions, it has been shown that the \textit{cfr} gene can also be expressed in \textit{Escherichia coli}.	extsuperscript{3} However, up to now little is known about the presence of the \textit{cfr} gene in Gram-negative bacteria. Only one report has described the presence of the \textit{cfr} gene in a naturally occurring \textit{Proteus vulgaris} isolate of porcine origin.\textsuperscript{9} Based on this finding, we carried out a survey to investigate the presence and the genetic environment of the \textit{cfr} gene in \textit{E. coli} in domestic animals.

**Materials and methods**

**Bacterial isolates and detection of florfenicol resistance genes**

A total of 1230 \textit{E. coli} isolates were collected from individual pigs, chickens and ducks in Shandong (n=491; pigs n=189, ducks n=77 and chicken n=225) and Sichuan provinces (n=739; pigs n=218, ducks n=66 and chicken n=455) during 2008–10. The antibiotic
usage records for these domestic animal farms indicated that florfenicol had been used commonly and extensively. The E. coli isolates were cultivated overnight at 37°C in brain heart infusion (BHI) broth (Luqiao, Beijing, China). Since little is known about the expression levels of the cfr gene in naturally occurring E. coli, all isolates were subjected to PCR screening for the cfr gene. For this, the DNA was prepared by boiling the E. coli isolates, and previously described primers were used for PCR.\textsuperscript{10} Since the cfr gene is often co-located with other florfenicol-resistant genes, the cfr-positive strain was further investigated by PCR for the genes floR, fexA and fexB.\textsuperscript{5,11,12}

Susceptibility testing

MIC values were determined by broth microdilution according to the recommendations given in CLSI document M100-S21. The reference strains S. aureus ATCC 29213 and E. coli ATCC 25922 served as controls.

Molecular analysis

S1 nuclease pulsed-field gel electrophoresis (PFGE) was performed to estimate the sizes of plasmids.\textsuperscript{13} The location of the cfr and floR genes on plasmids or in the chromosomal DNA was determined by Southern blotting. Plasmid DNA was extracted using a Qiagen plasmid extraction kit (Qiagen, Hilden, Germany). Plasmid DNA was introduced into E. coli J53 and E. coli DH10B by conjugation\textsuperscript{13} and electrotransformation,\textsuperscript{9} respectively. In addition, the regions flanking the cfr gene were sequenced by a modified random primer walking strategy as previously described.\textsuperscript{15} To determine the stability of the IS26 flanking segment in plasmid pEC-01, both in the original strain and in the transformants, inverse PCR was performed using primers cfrIF and cfrIR (complementary to locations inside the cfr gene).\textsuperscript{9} In addition, each of the IS26 flanking segments in the above-mentioned strains was amplified by using primers located in the tnpA and Jhp4 genes, left and right of the IS26 flanking segment, respectively.

Functional analysis

A DNA fragment that contained the cfr gene plus 947 bp of the upstream sequence, including an intact IS26, was amplified by PCR using the primer pair E-F (5'-GCTTACAGCTTGGCAGAAAC-3') and E-R (5'-GCTTACTGATTTGCTATTTGATAC-3'). This PCR product was digested with XbaI, and then cloned into the XbaI site of the chloramphenicol- and tetracycline-resistant E. coli DH10B. The cloned insert was confirmed by sequence analysis. The recombinant plasmid was introduced into E. coli DH10B and S. aureus RN4220 by electroporation.\textsuperscript{9} Transformants were selected on Luria–Bertani (LB) plates containing 10 mg/L chloramphenicol and further confirmed by PCR for the presence of the cfr gene. Subsequently, these transformants were investigated for their MICs of linezolid, florfenicol, tiamulin and clindamycin.

Rapid amplification of 5' cDNA ends (5' RACE) assay

Total bacterial RNA was isolated using the RNeasy Mini Kit (Qiagen). A SMARTer RACE cDNA Amplification kit (Clontech, Otsu, Japan) was used to produce cDNA synthesized from total bacterial RNA. The 5' RACE reaction was performed with a cfr-specific primer (GS1, 5'-GGTGCTTAG AGCTTACACCTATCCC-3') using a touchdown PCR protocol as described by the manufacturer. The PCR fragments were cloned into a PMD-19T vector (Takara, Dalian, China) and sequenced. Four separate positive clones were sequenced for accurate mapping of the transcription start site.

Results and discussion

The analysis of 1230 E. coli isolates revealed the presence of the cfr gene in a single isolate, designated LYP-C-BCTb11, obtained from the nasal swab of a pig in a slaughterhouse in Shandong province in 2010. Although information of antimicrobial therapy for this particular pig was not available, the antibiotic usage records of the farm in which the pig was fattened and from which it was transferred to the slaughterhouse indicated that a number of antimicrobial agents, including penicillin, florfenicol, trimethoprim/sulfamethoxazole, kanamycin, streptomycin, oxytetracycline and tylosin had been used for curing or preventing bacterial infections. This E. coli isolate carried both the floR and cfr-resistance genes as confirmed by sequence analysis. The nucleotide sequence of the cfr gene in isolate LYP-C-BCTb11 showed 100% identity to the cfr gene of the Staphylococcus sciuri plasmid pSCFS1 (accession no. NC_005076).\textsuperscript{16} Moreover, the 959 bp PCR product showed that the floR gene was indistinguishable from the floR gene on plasmid 10660-1 from E. coli (accession no. AF231986).\textsuperscript{17} Isolate LYP-C-BCTb11 exhibited high MIC values of chloramphenicol (256 mg/L), florfenicol (512 mg/L), gentamicin (128 mg/L), ampicillin (256 mg/L), ciprofloxacin (32 mg/L) and trimethoprim/sulfamethoxazole (>32/608 mg/L), but showed only low MIC values of ceftiofur (<0.25 mg/L) and ceftazidime (0.125 mg/L).

Conjugation using E. coli LYP-C-BCTb11 as a donor strain and E. coli J53 as a recipient strain failed, but electrotansformation using plasmids extracted from LYP-C-BCTb11 with E. coli DH10B as a recipient strain was successful. The sizes of the plasmids present in the E. coli isolate LYP-C-BCTb11 and its transformant DH10B-BCTb11 were determined by S1 nuclease-PFGE. The original strain harboured multiple plasmids, but only two plasmid bands were observed in the transformant (Figure 1a). Southern blot analysis with probes specific for cfr and floR identified the floR gene on a plasmid of ~194 kb and in the chromosomal DNA of LYP-C-BCTb1, whereas the cfr probe hybridized to a plasmid of ~110 kb, and this plasmid was designated pEC-01 (Figure 1b and c). Susceptibility testing showed that the transformant DH10B-BCTb11 conferred resistance not only to chloramphenicol (256 mg/L) and florfenicol (512 mg/L), but also to trimethoprim/sulfamethoxazole (32/608 mg/L). PCR screening for trimethoprim and sulfonamide resistance genes using previously described primers\textsuperscript{18,19} revealed the presence of the genes sul2 and dfrA17 in DH10B-BCTb11.

The genetic environment of the cfr gene was determined by a modified random primer walking strategy, starting from each end of the cfr gene in the transformant DH10B-BCTb11. A 12390 bp segment of pEC-01 containing the cfr gene was determined (Figure 2, accession no. JN982327). Within this plasmid segment, a 3849 bp element comprised a cfr-carrying central region of 1545 bp and this was flanked by IS26 insertion sequences in the same orientation. This cfr-carrying central region showed 100% identity to the corresponding region of plasmid pSCFS1 of S. sciuri.\textsuperscript{16} Immediately downstream of the left-hand IS26 was the transposase gene tnpA, which showed 100% identity with tnpA of the E. coli plasmid pEC49-1 (accession no. JN157839). However, no direct target site duplications were found immediately upstream or downstream of these IS26 elements. Interestingly, the flanking regions of this 3849 bp element showed high homology (>96%) to plasmid
pCROD2 (accession no. NC_010657) from *Citrobacter rodentium* ICC168\(^\text{20}\) and to plasmid pBS512_33 (accession no. NC_010657) from *Shigella boydii* CDC 3083-94 (Figure 2). The structural similarities between plasmid pEC-01 and plasmids pCROD2/pBS512_33 suggested that a 3849 bp element containing the gene \( cfr \), two IS\(^{26} \) elements and the gene \( tnpA \) had integrated into a pCROD2-like or a pBS512_33-like backbone. Previous studies have suggested that IS\(^{26} \) elements may play an important role in the mobility of \( cfr \).\(^{9} \) IS\(^{26} \) elements were also identified in close proximity to \( cfr \) in plasmid pEC-01. By using the inverse PCR with primers cfrIF and cfrIR, a 2192 bp amplicon was obtained, and sequence analysis confirmed that it contained an intact \( cfr \)-carrying central region and one intact IS\(^{26} \). In addition, two PCR amplicons were obtained by using primers located in the \( tnpA \) and \( \Delta hp4 \) genes (2228 and 4593 bp). Sequence analysis indicated that the smaller amplicon contained one complete IS\(^{26} \) and the flanking \( tnpA \) and \( \Delta hp4 \) sequences, while the 4593 bp amplicon comprised the intact IS\(^{26} \) flanking segment and the flanking \( tnpA \) and \( \Delta hp4 \) sequences. As previously shown for *P. vulgaris*,\(^{9} \) the PCR-based stability tests also revealed that a pEC-01 segment carrying the \( cfr \) gene and one IS\(^{26} \) copy can easily be excised.

Determination of the MICs of linezolid, tiamulin, clindamycin and florfenicol for *S. aureus* RN4220 and *E. coli* DH10B, each carrying the recombinant plasmid pAME2, confirmed the functionality of the \( cfr \) gene of *E. coli* LYP-C-BCTb11. *S. aureus* RN4220 harbouring pAME2 exhibited at least 4-fold elevated MICs of the tested antibiotics compared with the \( cfr \)-negative recipient strains (Table 1). Since the previously reported \( cfr \) promoter is truncated in this study,\(^{19} \) the location of the \( cfr \) promoter

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**Figure 1.** Identification of \( cfr \) and \( floR \) genes in the plasmids of *E. coli* LYP-C-BCTb11. (a) Plasmid size determination by S1 nuclease-PFGE, (b) Southern blot hybridization with a \( floR \)-specific probe and (c) Southern blot hybridization with a \( cfr \)-specific probe. Lane M1, Low Range PFG Marker (New England Biolabs, Beverly, MA, USA); lane 1, *E. coli* LYP-C-BCTb11; lane 2, transformant *E. coli* DH10B-BCTb11; lane 3, *E. coli* DH10B.

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**Figure 2.** Genetic environment of the \( cfr \) gene in plasmid pEC-01 of *E. coli* LYP-C-BCTb11, and structural comparison with plasmids pCROD2 from *Citrobacter rodentium* ICC168, pBS512_33 from *Shigella boydii* CDC 3083-94 and pSCFS1 from *S. sciuri*. The arrows indicate the positions and directions of transcription of the genes. Different genes are indicated by different shadings. The IS\(^{26} \) elements are shown as light grey boxes with the white arrows indicating the transposase (\( tnp \)) genes and the dark grey arrowhead indicating inverted repeats of IS elements. Regions of >96% homology are marked by grey shading. Black arrowheads indicate the primers used for amplifying fragments to construct pAME2.

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sequence was further analysed by mapping the cfr transcription start site using a 5’ RACE assay. The +1 position was identified 41 bp upstream of the cfr start codon, and the transcription site was located 6 bp downstream of the putative −10 sequence (TATAAT), while the putative −35 sequence (TTGCAA) was located 30 bp upstream of the −10 sequence. Interestingly, the −35 sequence of cfr was located within the terminal inverted repeat of the IS26 element. A similar situation has been described: the terminal base pairs of an IS256 element, which had become integrated into the translational attenuator of a staphylococcal erm(A) gene, restored the −35 sequence and thereby generated a hybrid promoter.21

In conclusion, this study showed that the staphylococcal multiresistance gene cfr was found in E. coli of domestic-animal origin, although at a very low prevalence (0.08%). The findings of the present study together with the previous report of cfr in a P. vulgaris isolate of porcine origin9 suggest that the detection of the cfr gene in Gram-negative bacteria of domestic-animal origin may represent only sporadic incidence. Continued surveillance of the presence of the cfr gene in Gram-negative bacteria of domestic-animal origin is warranted to monitor potential dissemination of this multiresistance gene.

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Transparency declarations
None to declare.

References


