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FRI-2 carbapenemase-producing *Enterobacter cloacae* complex in the UK

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Objectives: Detection of rarer carbapenemases is challenging, as it requires molecular assays with comprehensive coverage or the use of phenotypic methods for the detection of carbapenemase activity. We describe a new class A carbapenemase, FRI-2, in an *Enterobacter cloacae* complex isolate following implementation of an in-house multiplex PCR for the detection of 'rare' class A carbapenemases.

Methods: MICs were determined by agar dilution. A carbapenem-resistant *E. cloacae* complex isolate was tested by PCR for the class A carbapenemases bla_{KPC}, bla_{FRI}, bla_{IMI}, bla_{GES} and bla_{SME}. Carbapenemase activity was assessed using Carba NP and the carbapenem inactivation method. Whole genome and plasmid analyses of the clinical isolate and the FRI-2 transformant were performed by WGS, respectively. Typing was carried out by PFGE.

Results: The *E. cloacae* complex isolate showed resistance to imipenem (MIC = 16 mg/L), meropenem (MIC = 8 mg/L) and ertapenem (MIC = 8 mg/L), but remained susceptible to piperacillin/tazobactam (MIC = 8 mg/L). Carbapenemase activity was confirmed in the isolate by both phenotypic methods. A $bla_{FRI\text{-}1\text{-}like}$ gene was detected by PCR and analysis of WGS data of the clinical isolate identified an ORF of 885 bp, which showed 97% nucleotide identity with $bla_{FRI\text{-}1}$ and was named $bla_{FRI\text{-}2}$. WGS of the transformant indicated $bla_{FRI\text{-}2}$ was located on a 108 kb IncF/IncR plasmid. The FRI-2-positive *E. cloacae* complex isolate belonged to a novel ST (ST829).

Conclusions: The possible circulation of rarer carbapenemases in clinical settings highlights the role of phenotypic tests to detect carbapenemase activity when molecular assays are negative for the 'big 5' carbapenemase families.

Introduction

In Enterobacteriaceae, carbapenem resistance mediated by carbapenemases is mostly attributed to the interspecies dissemination of KPC, OXA-48-like, NDM, VIM and IMP alleles. Although the dispersion of the 'big 5' carbapenemases has been reported worldwide, some carbapenemases, such as bla_{SME} or bla_{SPM}, are still species- or, largely, country-specific.[1,2]

Class A carbapenemases other than KPC include the closely related NMC-A and IMI, SME, GES, BIC-1, SFC-1 and BKC-1.[3] The detection of these carbapenemases poses a problem in terms of infection prevention and control as most commercial and in-house molecular assays have limited coverage and focus on the detection of the 'big 5' families. Moreover, due to the rarity of these other carbapenemases and their potential susceptibility to cephalosporins, phenotypic tests could be misinterpreted as false-positives if further extended molecular characterization is not undertaken.[4]

Dortet *et al.*[5] recently identified a novel class A carbapenemase, FRI-1, in an isolate of *Enterobacter cloacae* in a patient in France. FRI-1 shares 53% and 42% identity with SFC-1 and BKC-1, respectively, and is related to a subgroup including SME-1, IMI-1 and NMC-A, with identity ranging from 54% to 56%.

PHE's Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit has recently implemented an in-house multiplex PCR for the detection of the 'rare' class A carbapenemases FRI, IMI, GES and SME. During screening of clinical isolates sent to the AMRHAI Reference Unit for investigation of carbapenem resistance, we identified a variant of FRI-1 in an *E. cloacae* complex strain, subsequently named FRI-2.

Materials and methods

An *E. cloacae* complex isolate 587 was submitted to PHE's AMRHAI Reference Unit in June 2016 for investigation of carbapenem resistance. This isolate was recovered from a patient who attended an outpatient clinic at the University Hospital of South Manchester (UK) and as part of routine assessment prior to a coronary artery bypass graft underwent rectal screening for carbapenemase-producing Enterobacteriaceae (CPE). Rectal screening for CPE using culture is routine for patients at higher risk of CPE carriage (including those admitted to hospitals in Greater Manchester in the previous year).[6] The patient had been admitted for cardiac problems in two hospitals in England including the referring hospital in the year before detection. The patient had no recent history of antibiotic administration or travel abroad. No previous CPE had been isolated from this patient, although there had been no previous rectal screening for CPE.

MICs were determined by agar dilution against AMRHAI's standard Gram-negative antibiotic panel, which includes imipenem (±EDTA for MBL detection), ertapenem and meropenem (±avibactam for class A carbapenemases detection), and interpreted using EUCAST criteria.[7]

Carbapenemase genes, including various class A carbapenemases (bla_{KPC}, bla_{FRI}, bla_{IMI}, bla_{GES}, bla_{SME}) were sought using in-house multiplex PCRs[8] with the following primers for the detection of genes encoding FRI-1 (FRI-1 For: 5′-AGG TGA ATT GGC GTC TGT CC-3′ and FRI-1 Rev: 5′-GGG GGC GGA TAG GTG TTT AT-3′), IMI (IMI-for: 5′-CTA CGC TTT AGA CAC TGG CTC-3′ and IMI-rev: 5′-TTG GTA CGC TAG CAC GAA TA-3′), GES (GES-F: 5′-TTT CTA GCA TCG GGA CAC AT-3′ and GES-R: 5′-ACA GCG TAA TCT CTC TCC TG-3′) and SME (SME-F: 5′-AAC GGC TTC ATT TTT GTT TAG-3′ and SME-R: 5′-GCT TCC GCA ATA GTT TTA TCA-3′) enzymes.

Evidence of carbapenemase activity was sought using Rapidec® Carba NP (bioMérieux SA, Marcy-l'Étoile, France) and the carbapenem inactivation method (CIM).[9]

Electroporation was performed using *Escherichia coli* Alpha-Select recipient cells (Bioline, London, UK). Transformants were selected on LB agar (Oxoid Ltd, Basingstoke, UK) containing 100 mg/L ampicillin (Sigma–Aldrich, Gillingham, Dorset, UK) and were screened for bla_{FRI-1} by PCR before WGS.

WGS was performed on both the clinical isolate and FRI-2 transformant using a Hiseq sequencer (Illumina) and the resulting data were analysed using an in-house bioinformatics pipeline; resistance genes were identified by mapping reads against a library curated in-house from publically accessible databases.[10] Transformant reads were assembled into contigs using SPAdes 3.5.0 (http://bioinf.spbau.ru/spades), with K-mer values of 21, 33, 55 and 77. Plasmid contigs were extracted from the known *E. coli* host genome and gaps were closed by PCR and subsequent Sanger sequencing. Plasmid replicon types were determined using PlasmidFinder[11] on the Center for Genomic Epidemiology platform (https://cge.cbs.dtu.dk/services/). Genes were inferred and annotated using PROKKA[12] and BLAST. ST was determined *in silico* (https://pubmlst.org/ecloacae/).[13]

Typing was carried out by PFGE of XbaI-digested genomic DNA.

Nucleotide sequence accession numbers

The nucleotide sequence data have been submitted to the GenBank nucleotide database under accession no. KX620467 for bla_{FRI-2} and KX912253 for the plasmid pJF-587.

Results and discussion

Resistance to carbapenems in *Enterobacter* spp. isolates is encountered frequently in the UK, but is mainly associated with a combination of several mechanisms including production of ESBL, increased efflux, porin alteration and/or overproduction of the intrinsic AmpC enzyme,[14,15] hence the difficulty in relating carbapenem resistance with carbapenemase production. Between 2004 and the end of December 2016, 3666 *Enterobacter* spp. isolates had been received in AMRHAI for investigation of carbapenem resistance with 1059 (28.9%) isolates confirmed as carbapenemase producers [KPC ($n = 349$), OXA-48-like ($n = 356$), NDM ($n = 198$), VIM ($n = 79$), IMP ($n = 38$), IMI ($n = 22$), GES-5 ($n = 6$) and the one *E. cloacae* producing FRI-2 reported here; 10 isolates co-produced two carbapenemases; K. L. Hopkins and N. Woodford, AMRHAI, unpublished data].

The *E. cloacae* complex isolate was resistant to imipenem (MIC = 16 mg/L), meropenem (MIC = 8 mg/L), ertapenem (MIC = 8 mg/L) and aztreonam (MIC = 32 mg/L), but remained susceptible to the cephalosporins (cefotaxime, 1 mg/L; ceftazidime, 0.5 mg/L; and cefepime, ≤0.125 mg/L) and to non-β-lactam antibiotics including colistin (MIC ≤0.5 mg/L), aminoglycosides (gentamicin, 0.25 mg/L; tobramycin, 0.5 mg/L; and amikacin, 1 mg/L) and ciprofloxacin (MIC ≤0.125 mg/L) (Table 1). Carbapenem resistance of the isolate with relative susceptibility to the cephalosporins and synergy observed between meropenem (MIC = 8 mg/L) and meropenem/avibactam (MIC = 0.03 mg/L; the concentration of avibactam is fixed at 4 mg/L) suggested production of a class A or D carbapenemase. The isolate remained susceptible to piperacillin/tazobactam (MIC = 8 mg/L) and temocillin (MIC = 4 mg/L), and KPC and OXA-48-like carbapenemases were excluded by PCR; however, Carba NP and CIM confirmed carbapenemase activity in the isolate.

An ampicillin-resistant transformant was resistant to aztreonam (MIC = 16 mg/L) and showed reduced susceptibility to ertapenem (MIC = 0.25 mg/L), meropenem (MIC = 0.125 mg/L) and imipenem (MIC = 4 mg/L) compared with the carbapenem MICs for the recipient strain (Table 1).

A $bla_{FRI-1-like}$ gene was detected by in-house multiplex PCR, and was confirmed by Sanger sequencing of the amplicon. Subsequent analysis of WGS data of the *E. cloacae* complex isolate identified an ORF of 885 bp, which showed 97% nucleotide identity with bla_{FRI-1}. The newly named bla_{FRI-2} was predicted to encode a 294 amino acid protein, which shares 275/294 (94%) amino acid identity with FRI-1 (Figure 1a). The other antibiotic resistance genes detected by WGS were bla_{ACT-2}, encoding a chromosomal AmpC enzyme, *fosA*, and a mutation in *gyrA* at position 83 (S→T). WGS of the transformant indicated bla_{FRI-2} was located on a 108 kb IncF/IncR plasmid, which did not share significant homology with any plasmid sequences in public databases. The LysR family regulator (FRI-R) was present downstream of bla_{FRI-2} and shared 95.8% identity with FRI-R described downstream of bla_{FRI-1}; the IS*Ec8* element described upstream of bla_{FRI-1} was absent.[5] Downstream of bla_{FRI-2}, a MerR family transcriptional regulator was identified where IS*Ec14* was previously characterized near bla_{FRI-1} (Figure 1b). One hundred and nineteen ORFs were identified on the 108 kb plasmid with 69 ORFs showing no similarity with published sequences. The remaining ORFs encoded for genes involved in plasmid conjugation and partitioning; no other resistance genes or any virulence genes were encoded on the plasmid.

The G + C content for both bla_{FRI-2} and bla_{FRI-1} was similar (39%); however, the plasmid pJF-587 bearing bla_{FRI-2} had a G + C content of 52% suggesting horizontal acquisition of the gene.

The first reported FRI-1-producing *E. cloacae* complex strain was recovered from a urine sample of a patient hospitalized in

Table 1. MICs of different antibiotics for *E. cloacae*, *E. coli* Alpha-Select transformed with pJF-587 and *E. coli* Alpha-Select

	MIC (mg/L)		
Antibiotic	*E. cloacae*	pJF-587 *E. coli* Alpha-Select (FRI-2)	*E. coli* Alpha-Select
Ertapenem	8	0.25	≤0.125
Imipenem	16	4	0.25
Imipenem/EDTA	8	2	0.125
Meropenem	8	0.125	≤0.060
Meropenem/avibactam (4 mg/L)	0.03	0.015	0.015
Cefotaxime	1	≤0.125	≤0.125
Cefotaxime/clavulanate	0.25	≤0.060	≤0.060
Ceftazidime	0.5	0.25	≤0.125
Ceftazidime/clavulanate	0.25	0.125	0.125
Ceftazidime/avibactam	0.25	0.125	0.25
Cefepime	≤0.125	≤0.125	≤0.125
Aztreonam	32	16	≤0.125
Temocillin	4	8	4
Piperacillin/tazobactam	8	≤1.0	≤1.0
Ciprofloxacin	≤0.125	≤0.125	≤0.125
Gentamicin	0.25	0.25	0.25
Tobramycin	0.5	0.25	0.25
Amikacin	1	1	≤0.5
Tigecycline	0.5	≤0.250	≤0.250
Colistin	≤0.5	≤0.5	≤0.5
Carba NP	+	NT	NT
CIM	+	NT	NT

NT, not tested.

France and who had travelled to Switzerland.[5] The UK patient had no travel history, and the only risk factor potentially associated with acquiring the FRI-2-positive *E. cloacae* complex strain was previous hospitalization in two hospitals in North West England. PFGE typing of the *E. cloacae* failed due to DNA degradation so no comparison with *E. cloacae* isolates from either of the hospitals attended by the patient could be performed. The WGS data analysis showed that the FRI-2-positive *E. cloacae* isolate belonged to a novel ST (ST829).

To the best of our knowledge, this is only the second report globally of a FRI family carbapenemase. FRI-2 has substantial sequence divergence from FRI-1 (6% amino acid differences), and may therefore represent a distinct gene 'escape' event from an unidentified source species. Other class A carbapenemases previously identified in the *Enterobacter* genus include KPC and rarer carbapenemases, such as IMI and GES. The identification of bla_{IMI} in *E. coli* and *Klebsiella variicola* demonstrates that despite the rare isolation of such carbapenemases, they have the potential to transfer within and between species.[16,17] The presence of ISs and the location of FRI-2 on a plasmid highlight this potential. The identification of FRI-2 in a clinical setting in the UK demonstrates the role of phenotypic tests to detect carbapenemase activity. When molecular assays are negative for the 'big 5' carbapenemase families, microbiologists should consider the possibility of other carbapenemase types that are currently rare, but have the potential to become more significant public health problems.

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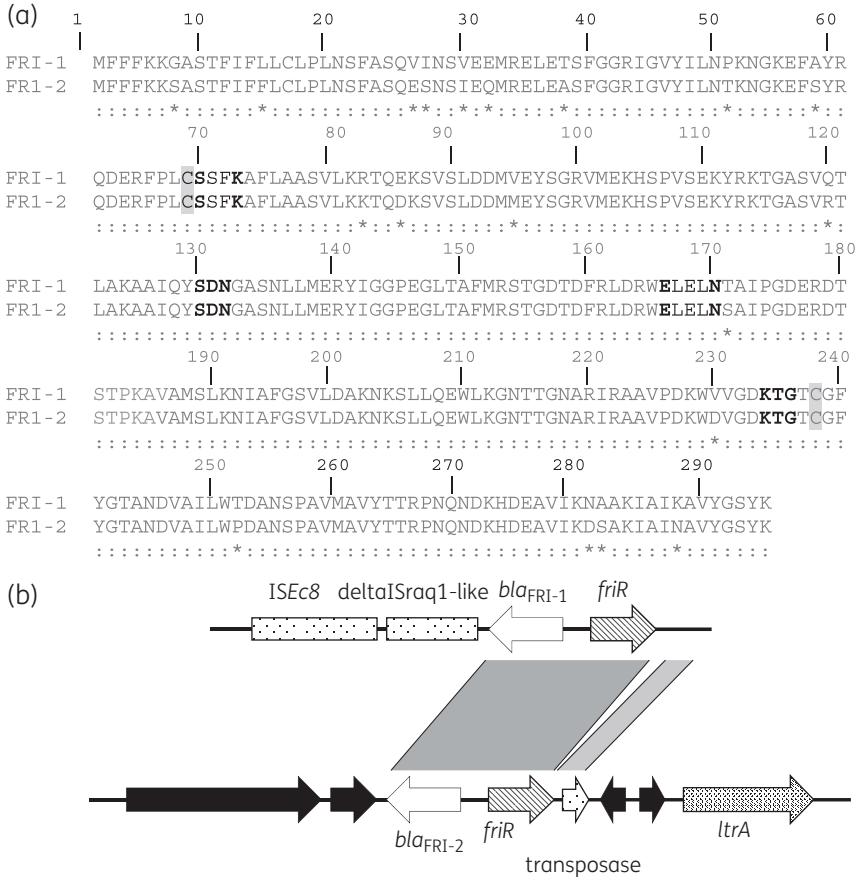

Figure 1. (a) Comparison of the amino acid sequences of FRI-1 and FR1-2. The conserved active-site motifs characteristic of class A β-lactamases ^{70}SXX^{73}K, ^{130}SD^{132}N, ^{166}EXXX^{170}N and ^{234}KT^{236}G (class A β-lactamase numbering scheme) are indicated in bold. Cysteine residues ^{69}C and ^{238}C involved in the disulphide bond are highlighted in grey. Asterisks indicate the amino acid differences. (b) Comparison of the genetic environments of bla_{FRI-1} and bla_{FRI-2} genes.

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