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Detection and epidemiology of plasmid-mediated AmpC β-lactamase producing *Escherichia coli* in two Irish tertiary care hospitals

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ABSTRACT

This study determined the prevalence and distribution of plasmid-mediated AmpC (pAmpC) β-lactamases in Irish *Escherichia coli* isolates. Clinical *E. coli* isolates ($n = 95$) that were intermediate or resistant to cefoxitin and/or flagged by VITEK® 2 as potential AmpC-producers underwent confirmation using a MASTDISCS™ ESBL and AmpC Detection Kit. Multiplex PCR capable of detecting family-specific plasmid *ampC* genes was performed to detect the presence of these genes. Five PCR-negative isolates were selected for promoter analysis. PFGE and MLST were performed on *E. coli* isolates that harboured a plasmid *ampC* gene to determine their clonal relatedness. Plasmid *ampC* genes were detected in 19% (18/95) of phenotypic AmpC producing *E. coli* isolates. The CIT group was the most common plasmid family type ($n = 14$); DHA ($n = 3$) and ACC ($n = 1$) groups were also detected. Promoter analysis showed that four isolates had multiple point mutations and one had a 1 bp insertion in the −10 box. PFGE demonstrated a polyclonal pattern for *E. coli* isolates. Furthermore, with the exception of two isolates with an identical sequence type (ST720), MLST analysis revealed that these isolates were not clonally related. This study revealed that there was a marked prevalence of pAmpC *E. coli* among phenotypic AmpC producing *E. coli* isolates but no evidence of cross-transmission of a single strain. Establishing the prevalence and clonality of these organisms is important in order to implement evidence-based infection control measures that reduce the spread of pAmpC β-lactamase resistance in the hospital environment.
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1. Introduction

Antibiotic resistance among *Enterobacteriaceae* is an ever-increasing concern both in Ireland and worldwide. Infection with antibiotic-resistant *Enterobacteriaceae* can lead to treatment failures due to limited antibiotic treatment options as well as posing the risk of cross-infection. AmpC β-lactamase producing organisms hydrolyse narrow-, broad- and expanded-spectrum cephalosporins and cephamycins (e.g. cefoxitin) and are usually only susceptible to fourth-generation cephalosporins and carbapenems [1]. AmpC β-lactamase resistance may be conferred through chromosomal or plasmid-mediated origins.

The chromosomal *ampC* gene in *Escherichia coli* is generally expressed at low levels, not causing clinical resistance, because the promoter is weak and the gene contains a transcriptional attenuator [2]. However, constitutive overexpression of *ampC* in *E. coli* can occur due to either the hyperproduction of a chromosomally encoded *ampC* gene or by acquisition of a transferable *ampC* gene on a plasmid [3]. The occurrence of *ampC* β-lactamases on plasmids presents the threat of spreading this resistance mechanism to other organisms within a hospital or geographic region.

Ireland started reporting third-generation cephalosporin sensitivity among *E. coli* in 2002. Between 2002 and 2010, the proportion of resistant *E. coli* increased from 2.2% to 7.7%. There is limited information with regard to the prevalence and epidemiology of plasmid-mediated AmpC (pAmpC) β-lactamases among *E. coli* in Ireland. Roche et al. reported that 3% of *Klebsiella pneumoniae* collected prospectively from April 2005 to March 2007 carried

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pAmpC in Ireland [4]. A study undertaken by the UK Antibiotic Resistance Monitoring and Reference Laboratory in 2007 reported a prevalence rate of pAmpC of 49% (67/135) and 55% (21/38) among cephalosporin- and cefoxitin-resistant *E. coli* and *Klebsiella* spp., respectively [5]. It is important to identify the occurrence of pAmpC in these organisms in order to implement necessary infection control measures.

The aim of this study was to establish the prevalence and clonality of clinical isolates of pAmpC producing *E. coli* in two south Dublin hospitals. *E. coli* isolates that were intermediate or resistant to cefoxitin and/or were flagged by the VITEK® 2 system as potential AmpC-producers underwent a phenotypic test to confirm the production of an AmpC β-lactamase. Multiplex PCR was subsequently carried out to identify the presence of plasmid *ampC* genes among isolates that demonstrated an AmpC phenotype. Finally, the genetic relatedness of pAmpC producing *E. coli* isolates was investigated through pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

2. Methods

2.1. Bacterial strains and antimicrobial susceptibility testing

A total of 95 non-duplicate AmpC producing *E. coli* isolates were collected from two hospitals in Dublin (Ireland) from March 2011 to October 2012.

Identification and susceptibility testing were performed by the VITEK® 2 Compact (bioMérieux, Marcy-l'Étoile, France) and the results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints v.2.0 and EUCAST Expert rules v.1.0 [6]. The advanced expert system (AES) of the VITEK® 2 Compact identified the isolates as potential AmpC producers on the basis of an antimicrobial susceptibility pattern that is compatible with an AmpC producing phenotype, i.e. resistance to first- and second-generation cephalosporins, resistance to one or more of the third-generation cephalosporins and/or reduced susceptibility to cefoxitin, and sensitivity to the fourth-generation cephalosporin cefepime.

2.2. Phenotypic AmpC β-lactamase detection

Isolates were confirmed phenotypically as AmpC β-lactamase producers by the MASTDISCS™ ESBL and AmpC Detection Kit (MAST Group Ltd., Merseyside, UK). The MASTDISCS™ kit is an inhibitor-based test that detects the presence of AmpC β-lactamases and extended-spectrum β-lactamases (ESBLs). Briefly, a Mueller–Hinton agar plate (L.I.P. Diagnostic Services, Galway, Ireland) was inoculated with a 0.5 McFarland standard suspension of a test isolate. The A (10 μg cefpodoxime), B (10 μg cefpodoxime + the ESBL inhibitor clavulanic acid), C (10 μg cefpodoxime + the AmpC inhibitor cloxacillin) and D (10 μg cefpodoxime + ESBL and AmpC inhibitors) disks were applied onto the plate. The zone of inhibition around each disc was measured following 18–24 h of incubation at 37 °C in air and was interpreted according to the manufacturer's instructions.

2.3. Multiplex PCR

Isolates confirmed as AmpC β-lactamase producers by the phenotypic MASTDISCS™ ESBL and AmpC Detection Kit were screened for the presence of a plasmid-borne *ampC* gene using a multiplex PCR assay developed by Pérez-Pérez and Hanson [7]. This PCR utilises six primer sets to detect the six phylogenetic families of plasmid *ampC* β-lactamase genes (i.e. bla_{FOX}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{MOX} and bla_{ACC}) on the basis of amplicon size and was performed as described previously [7]. A representative group of bla_{CIT} PCR products ($n = 7$) were randomly selected for sequencing. Sequence alignments and analysis were performed using the NCBI BLAST programme (http://www.ncbi.nlm.nih.gov).

2.4. Promoter analysis

Five of the multiplex PCR negative isolates were analysed for *ampC* promoter mutations, which included promoter PCR, sequencing of the products and multiple sequence alignment with the *E. coli* K12 strain promoter sequence [8].

2.5. Pulsed-field gel electrophoresis

Agarose-embedded plugs containing intact bacterial DNA were prepared using a CHEF (Contour-Clamped Homogenous Electric Fields) Mapper Plug Kit (Bio-Rad Laboratories, Hemel Hempstead, UK) according to the manufacturer's instructions. Following overnight digestion of the plugs with *Xba*I at 37 °C, PFGE was performed on a CHEF Mapper XA PFGE System (Bio-Rad Laboratories) according to the PulseNet standardised laboratory protocol [9]. The electrophoresis conditions were optimised for separation of the 30–600 kb fragments in a 1% agarose gel with 0.5× TBE [Tris–borate–ethylene diamine tetra-acetic acid (EDTA)] buffer and a voltage of 6 V/cm for 19 h, with an initial switch time of 2.2 s and final switch time of 54.2 s. A dendrogram was constructed using the Dice coefficient with an optimisation of 1% and a tolerance of 1% in band position difference (FPQuest™ Software; Bio-Rad Laboratories). The clonal relationship among isolates was determined according to the criteria of Tenover et al. [10]. Clonal groups were assigned based on a similarity of ≥80% (≤3 band difference in restriction profile).

2.6. Multilocus sequence typing

Nine *E. coli* isolates harbouring a plasmid *ampC* gene were selected for further characterisation through the *E. coli* MLST scheme developed by the Achtman group [11]. The protocol for this typing method, including the PCR primers, PCR components and cycling conditions required, was accessed through the online MLST *E. coli* database [12]. The PCR products of the seven housekeeping genes on all nine *E. coli* isolates were subsequently purified for sequencing using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Sanger sequencing was performed by an external laboratory (Source BioScience, LifeSciences, Dublin, Ireland) and the MLST PCR primer pairs were used for the sequencing step. The sequencing data of each isolate were entered into the *E. coli* MLST online database (http://mlst.ucc.ie/mlst/dbs/Ecoli) to give a distinct allelic number. The alleles for the seven loci were combined to give an allelic profile or sequence type (ST). The STs of each of the nine isolates were collated and compared.

3. Results

3.1. Antimicrobial susceptibility testing and MASTDISCS™

Ninety-five *E. coli* isolates were identified by the VITEK® 2 system as potential AmpC producers and were phenotypically confirmed for AmpC production using combination disks. One isolate was positive for the coexistence of AmpC and ESBL enzymes. *E. coli* isolates demonstrated variable resistance to the third-generation cephalosporins, with some isolates only resistant to one of either cefotaxime or ceftazidime or sensitive to both. Isolates that were positive for a plasmid *ampC* gene generally had higher minimum inhibitory concentrations (MICs) against the third-generation cephalosporins than chromosomally mediated AmpC producing isolates (Table 1). A total of 94% (17/18) and 83% (15/18) of pAmpC isolates were resistant to ceftazidime and

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Table 1
Minimum inhibitory concentrations (MICs) (in mg/L) of *Escherichia coli* isolates with an AmpC phenotype.

Antibiotic	pAmpC (n = 18)		Chromosomal AmpC (n = 77)	
	Mode MIC	MIC range	Mode MIC	MIC range
AMP	≥32	≥32	≥32	≥32
CXM	≥64	16 to ≥64	16	4 to ≥64
CTX	16	≤1 to ≥64	≤1	≤1 to 16
CAZ	8	≤1 to ≥64	≤1	≤1 to ≥64
FEP	≤1	≤1–2	≤1	≤1
FOX	≥64	8 to ≥64	16	≤4 to ≥64
MEM	≤0.25	≤0.25	≤0.25	≤0.25
ETP	≤0.5	≤0.5–1	≤0.5	≤0.5
GEN	≤1	≤1 to ≥16	≥1	0.25 to ≤16
CIP	≤0.25	≤0.25 to ≥4	≤0.25	≤0.25 to ≥4

pAmpC, plasmid-mediated AmpC; AMP, ampicillin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; MEM, meropenem; ETP, ertapenem; GEN, gentamicin; CIP, ciprofloxacin.

cefotaxime, respectively. In contrast, chromosomally mediated AmpC producing isolates were more sensitive, with only 12% (9/77) resistant to ceftazidime and 14% (11/77) resistant to cefotaxime. With the exception of one isolate (E25), AmpC-producing *E. coli* isolates in this study were resistant to cefoxitin. Most isolates (n = 94) were sensitive to the fourth-generation cephalosporin cefepime, except for one isolate (E23) that was also non-susceptible to ertapenem. All 95 isolates were sensitive to meropenem. In addition, many isolates demonstrated multi-resistance; 16 isolates displayed resistance to the aminoglycoside gentamicin and 31 isolates were resistant to the fluoroquinolone ciprofloxacin.

3.2. AmpC multiplex PCR

Of the 95 isolates that were screened for the presence of a plasmid *ampC* β-lactamase gene by multiplex PCR, 18 isolates (19%) were pAmpC positive. The predominant gene was bla_{CIT}, detected in 14 *E. coli* isolates (Table 2). Nucleotide sequencing of a representative group of these isolates (n = 7) revealed 100% concordance with the bla_{CMY-2} gene sequence (GenBank accession no. HQ680723). Other pAmpC genes identified were bla_{DHA} (n = 3) and bla_{ACC} (n = 1). The majority of phenotypic AmpC producing *E. coli* isolates were negative for a pAmpC gene, which suggests that the presence of AmpC could be due to either hyperproduction of chromosomal AmpC or acquisition of novel plasmids that were not detected by the multiplex PCR in this study.

3.3. Promoter analysis

Five of the multiplex PCR negative isolates were analysed for *ampC* promoter mutations. Four of them had multiple point mutations in the promoter region and one had a 1-bp insertion in the −10 box compared with *E. coli* K12 (Table 3). The predominant point mutation was at +58, followed by the −35 box.

3.4. Isolates with plasmid-mediated AmpC are genetically unrelated

The 18 *E. coli* isolates harbouring plasmid *ampC* genes were not clonally related by PFGE analysis and revealed <50% sequence homology. Furthermore, a representative group of nine pAmpC positive *E. coli* isolates were typed by MLST. With the exception of two isolates (isolates 90 and 91), which were ST720, MLST analysis revealed that these isolates were unrelated (Table 2). Apart from ST493 and ST3672, all of the MLST types have been previously detected both in humans and livestock in Europe [12]. ST493 has been isolated from humans in Japan and ST3672 from wild animals in China [12].

4. Discussion

Antimicrobial resistance among *Enterobacteriaceae* is fast becoming a worldwide public health concern, and AmpC β-lactamases are an emerging group of antimicrobial resistance determinants [13]. The occurrence of *ampC* genes on plasmids presents a great challenge to infection control because plasmid-mediated genes can spread to other organisms within a hospital or geographic region. The lack of a widely accepted phenotypic AmpC β-lactamase detection method greatly hinders accurate detection and surveillance of AmpC producers and hence the ability to limit the spread of pAmpC resistance. This study was designed to determine the prevalence and clonality of pAmpC producing *E. coli* in two Dublin hospitals.

Table 2
Antimicrobial susceptibility and sequence types (STs) by multilocus sequence typing of plasmid-mediated AmpC-producing *Escherichia coli* (n = 18).

Isolate	MIC (mg/L)										AmpC multiplex PCR (plasmid bla_{AmpC})	ST
	AMP	CXM	CAZ	CTX	FOX	FEP	MER	ETP	GEN	CIP		
E05	>32	16	4	8	>64	≤1	≤0.25	≤0.5	>16	>4	CIT	NT
E16	>32	>64	>64	>64	32	≤1	≤0.25	≤0.5	>16	>4	CIT	NT
E18	>32	16	4	4	32	≤1	≤0.25	≤0.5	≤1	>4	CIT	NT
E19	>32	>64	>64	>64	>64	≤1	≤0.25	≤0.5	≤1	0.5	CIT	NT
E23	>32	>64	>64	>64	>64	2	≤0.25	1	≤1	≤0.25	CIT	NT
E25	>32	32	32	8	8	≤1	≤0.25	≤0.5	≤1	>4	ACC	NT
E27	>32	16	4	≤1	>64	≤1	≤0.25	≤0.5	>16	≤0.25	DHA	NT
E35	>32	>64	>64	16	>64	≤1	≤0.25	≤0.5	>16	>4	CIT	NT
E53	>32	>64	>64	32	>64	≤1	≤0.25	≤0.5	≤1	≤0.25	CIT	NT
E90	>32	>64	>64	16	>64	≤1	≤0.25	≤0.5	≤1	≤0.25	CIT	ST720 (35-3-58-6-5-16-4)
E91	>32	>64	≤1	≤1	>64	≤1	≤0.25	≤0.5	8	≤0.25	CIT	ST720 (35-3-58-6-5-16-4)
E113	>32	>64	4	4	>64	≤1	≤0.25	≤0.5	≤1	>4	DHA	ST38 (4-26-2-25-5-5-19)
E130	>32	32	16	8	>64	≤1	≤0.25	≤0.5	≤1	≤0.25	CIT	ST3672 (76-13-190-13-17-30-25)
B6	>32	16	16	4	>64	≤1	≤0.25	≤0.5	≤1	>4	CIT	ST10 (10-11-4-8-8-8-2)
B7	>32	>64	16	≤1	>64	≤1	≤0.25	≤0.5	>16	≤0.25	DHA	ST493 (40-13-9-13-16-10-9)
B10	>32	16	16	8	>64	≤1	≤0.25	≤0.5	≤1	≤0.25	CIT	ST92 (40-14-19-36-23-11-10)
B12	>32	32	16	8	>64	≤1	≤0.25	≤0.5	≤1	≤0.25	CIT	ST906 (6-4-3-16-11-8-6)
B18	>32	>64	16	8	>64	≤1	≤0.25	≤0.5	≤1	>4	CIT	ST354 (85-88-78-29-59-58-62)

MIC, minimum inhibitory concentration; AMP, ampicillin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; MEM, meropenem; ETP, ertapenem; GEN, gentamicin; CIP, ciprofloxacin; NT, not tested.

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Table 3
Mutations identified in the *Escherichia coli ampC* promoter region.

Isolate number	Mutations[a]
E7	−88 (C→T), −82 (A→G), −18 (G→A), −1 (C→T), +58 (C→T)
E10	−35 box (T→A), +58 (G→T), +63 (T→C)
E31	+70 (C→T) Attenuator: +21 (C→T), +25 (T→G), +26 (A→T), +31 (G→A)
E36	−35 box (T→A), +58 (C→T), +63 (T→C)
E46	1 bp (T) insertion in the −10 box

[a] Compared with *E. coli* K12.

A total of 95 *E. coli* clinical isolates phenotypically identified as AmpC β-lactamase producers by VITEK® 2 and combination disks were included in this study. Whilst the majority of them were identified as possible chromosomal AmpC hyperproducers ($n = 77$), a total of 18 isolates (19%) were pAmpC producers. This is compared with a study undertaken by the UK antimicrobial reference laboratory including Irish isolates that reported a prevalence rate of pAmpC of 49% (67/135) among cephalosporin-resistant *E. coli* spp. [5]. The higher prevalence rate detected in the UK study could potentially be explained by the fact that microbiology laboratories would only send the most resistant isolates for further analysis to reference laboratories, whereas in the current study serial clinical isolates exhibiting an AmpC phenotype were collected. Other studies carried out in hospital laboratories and similar in design to this study have revealed various prevalence rates. For example, a study from Denmark reported a similar prevalence to our data, in which 17% (4/24) of *E. coli* carried a plasmid *ampC* gene [14], whilst a study in Switzerland reported a higher prevalence at 38% (8/21) [15].

The predominant *ampC* gene detected in this study was bla_{CIT}, identified in 14 *E. coli* isolates. Nucleotide sequencing of a representative group of these isolates ($n = 7$) revealed the bla_{CMY-2} gene. This observation is consistent with many other studies worldwide in which CMY-2 is the most commonly acquired plasmid and widely geographically distributed AmpC enzyme [5,15–22].

In addition, bla_{DHA} ($n = 3$) and bla_{ACC} ($n = 1$) were also detected. The CIT, DHA and ACC AmpC enzymes have been previously identified among *E. coli* and *K. pneumoniae* isolates in Ireland [4,5]. The isolate (E25) that harboured a bla_{ACC} gene in this study was susceptible to cefoxitin, which correlates with many studies showing that isolates with ACC enzyme are sensitive to cefoxitin [4,23]. It is important to consider that the use of cefoxitin resistance as a screening marker may be compromised by isolates producing plasmid-encoded ACC AmpC β-lactamases that are sensitive to cefoxitin.

This study revealed that pAmpC producing *E. coli* isolates displayed higher MICs to the third-generation cephalosporins and cefoxitin than chromosomal AmpC hyperproducing *E. coli* (Table 1). Concomitant resistance to cefotaxime, ceftazidime and cefoxitin in phenotypic AmpC-producing *E. coli* isolates was suggestive of pAmpC production. Hence, whilst routine molecular testing for pAmpC in *E. coli* is currently unavailable in Ireland, the susceptibility profile of a phenotypically confirmed AmpC producing *E. coli* may help to distinguish between pAmpC production and chromosomal AmpC hyperproduction.

Co-resistance to other antimicrobials was frequently observed among these isolates. For example, ciprofloxacin resistance was observed in 33% (31/95) of AmpC producers in this study. A rising trend of fluoroquinolone resistance among AmpC producing *E. coli* has been reported by Simner et al. [3].

The coexistence of multiple resistance mechanisms in an organism is a concern as multidrug resistance can severely limit therapeutic options. In this study, co-production of AmpC and ESBL was detected in one isolate. Continuous resistance surveillance and the implementation of infection control measures are important to reduce the spread of multidrug-resistant organisms. Five of the multiplex PCR negative isolates were analysed for *ampC* promoter mutations and the results suggested that they are common mutations as they were also identified by Haldorsen et al., who reported three different mechanisms for increased expression of chromosomal *ampC* genes including multiple point mutations (positions −1, −18, −42, −82, −88 and +58) and single nucleotide or insertion sequence (IS) element insertions between the −35 and −10 boxes [16]. Further work is required to investigate the role of *ampC* promoter mutations in AmpC hyperproduction.

Detection of a plasmid *ampC* gene in an AmpC producing organism provides a valuable insight into the resistance mechanism of an isolate and the potential for this resistance to spread. It is equally important to understand whether antimicrobial resistance is due to the transmission of resistance genes from organism to organism or through the transmission of a single resistant strain. Molecular typing by PFGE and MLST was performed in this study to investigate whether pAmpC isolates were clonally related and hence the result of the spread of a single strain. PFGE showed that the pAmpC producers ($n = 18$) represented different DNA restriction patterns with <50% fragments sharing homology. These results suggest that the incidence of transferable AmpC in *E. coli* was the result of a mobile element rather than the spread of a single clone.

MLST was carried out on a representative group ($n = 9$) of pAmpC *E. coli* isolates in this study. MLST was performed due to the worldwide clonal dissemination of *E. coli* ST131 and recent detection of pAmpC β-lactamases among *E. coli* ST131 [24,25]. Among the nine selected *E. coli* isolates, MLST identified eight different STs. The pandemic *E. coli* clone ST131 was not detected in this study. Two isolates (isolates E90 and E91) had the same allelic profiles and therefore the same ST (ST720). These two isolates only displayed 40% similarity by PFGE, highlighting that MLST has a lower discriminating ability compared with PFGE, as reported in previous studies [26–28]. The remaining seven isolates had different individual allelic profiles and therefore different STs. The ST38 isolate in this study (isolate E113) carried a bla_{DHA} plasmid *ampC* gene. However, in two separate studies, pAmpC *E. coli* isolates related to the sequence type complex (STC) 38 instead harboured a bla_{CIT} plasmid *ampC* gene [24,25]. This illustrates how plasmids can spread among isolates and also that this globally distributed *E. coli* strain can acquire different plasmid *ampC* genes. Other strains identified in this study (ST92, ST493, ST720, ST906 and ST3672) have not been previously reported among pAmpC producing *E. coli* in the literature and could indicate that there has been further spread of plasmid *ampC* genes among *E. coli* strains.

In conclusion, this study revealed that there was a prevalence of 19% of pAmpC producing *E. coli* among isolates displaying an AmpC phenotype in two Dublin hospitals. The fact that the isolates investigated by PFGE and MLST were not clonally related demonstrates that pAmpC resistance was due to the transfer of plasmids among isolates rather than the spread of a single organism. As both hospitals do not routinely isolate patients harbouring *E. coli* with an AmpC phenotype, the lack of evidence for cross-infection by PFGE was reassuring. These findings emphasise the importance of investigating AmpC β-lactamases in clinical isolates at a molecular level, although routine molecular testing is currently beyond the scope of Irish diagnostic laboratories. In this way, informed and evidence-based infection control polices can be introduced to reduce the spread of pAmpC resistance in hospitals and limit the movement of this resistance mechanism.

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Competing interests

None declared.

Ethical approval

Not required.

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