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Short Communication

Emergence of NDM-5-producing *Escherichia coli* sequence type 167 clone in Italy

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ABSTRACT

The emergence of carbapenemase-producing Enterobacteriaceae (CPE) is a critical concern worldwide. In Italy, CPE isolates are very frequent, with the KPC enzyme types strongly predominant whereas the New Delhi metallo-β-lactamase (NDM) enzymes are extremely rare. Here we report the first detection of NDM-5-producing *Escherichia coli* sequence type 167 (ST167) isolates from two patients with urinary tract infection (Ec001 and Ec002 from urines), including one with colonisation (Ec003 from faeces) admitted to the same hospital 2 months apart in 2017. Minimum inhibitory concentrations (MICs) were determined by broth microdilution. The carbapenemase type was identified both by phenotypic and genotypic methods. Isolate genotypes were investigated by phylogenetic typing, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). Next-generation sequencing (NGS) was used to obtain complete sequences of plasmids. The three *E. coli* isolates carried the bla_{NDM-5} gene, shared the same resistance phenotype and belonged to ST167. By PFGE, isolates showed the same profile, suggesting that they were the same strain. NGS revealed that the bla_{NDM-5} gene was located on a 99-kb multireplicon plasmid (designed pNDM-5-IT) with a peculiar scaffold constituted by four replicons of the IncF type (FIA, FIB and two copies of the FII replicon). pNDM-5-IT plasmid harboured multiple resistance and virulence determinants, including the arginine deaminase (ADI) cluster never found associated with plasmids before. Since NDM-5-producing *E. coli* ST167 has been regarded as a successful epidemic clone in China, the emergence of such a clone carrying a plasmid associated both with multiresistance and virulence could be a public-health threat.

1. Introduction

The emergence and spread of carbapenemase-producing Enterobacteriaceae has become a worldwide public-health concern, although the geographical distribution of the different carbapenemase types varies considerably [1]. In Italy, the predominant carbapenemase is the KPC type disseminated especially among *Klebsiella pneumoniae* isolates; among the metallo-β-lactamase enzymes, VIM is found both in *K. pneumoniae* and *Escherichia coli*, whilst New Delhi metallo-β-lactamase (NDM) enzymes have rarely been detected [2–5]. In contrast, NDM-producing Enterobacteriaceae are abundant in the Indian subcontinent, the Balkans and Middle East regions, which act as a major reservoir [6]. Transferable plasmids carried by different clones of Enterobacteriaceae species mediate the geographical spread of NDM enzymes in non-endemic countries [7].

Among the Enterobacteriaceae, extraintestinal *E. coli* is the most prevalent pathogen causing both community- and healthcare-associated infections. Acquisition of NDM enzymes by *E. coli* isolates is a matter of concern since it would strongly limit the therapeutic options in a species that frequently carries multiresistance determinants. Knowledge regarding bla_{NDM} gene-harbouring clones and plasmids is important to understand the epidemiology of resistance and to control the spread of NDM-positive *E. coli* in the community and healthcare system and to monitor the dissemination of bla_{NDM} genes among other enterobacterial species.

Here we report the emergence of an NDM-5-producing *E. coli* sequence type 167 (ST167) clone in Italy. The total plasmid content of the NDM-producing ST167 isolates and the complete sequence of a novel bla_{NDM-5}-harboring plasmid (pNDM-5-IT) were determined.

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2. Materials and methods

2.1. Strain identification, antimicrobial susceptibility testing and characterisation of resistance genes

Routine identification to species level and antimicrobial susceptibility testing were performed by automated methods (VITEK®2; bioMérieux Italia S.p.A., Florence, Italy). Preliminary detection of NDM-type enzyme was carried out using the VERIGENE® Gram-negative Blood Culture (BC-GN) Test (Luminex, Hertogenbosch, The Netherlands). Antimicrobial susceptibility was confirmed by the reference broth microdilution method using the TREK Sensititre™ custom panel ITGNEGF (Thermo Fisher TREK Diagnostic Systems, Inc., Cleveland, OH). The interpretative breakpoints were based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) [8]. Phenotypic confirmatory testing for carbapenemase production was performed using agar tablet/disk diffusion method using the KPC/MBL and OXA-48 Confirm Kit (Rosco Diagnostica A/S, Taastrup, Denmark). Identification of carbapenemase-encoding genes (bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{OXA-48} and bla_{KPC}) and their variants was performed by PCR and sequencing [9]. Detection and sequencing of extended-spectrum β-lactamase (ESBL) and/or plasmid AmpC genes (bla_{CTX-M}, bla_{SHV}, bla_{TEM} and bla_{CMY-2}) was carried out as previously described [10].

2.2. Genotyping

NDM-producing *E. coli* isolates were assigned to one of the seven major *E. coli* phylogenetic groups (A, B1, B2, C, D, E and F) by the multiplex PCR-based method described by Clermont et al. [11]. Genetic relatedness among isolates was assessed by multilocus sequence typing (MLST) according to the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and by pulsed-field gel electrophoresis (PFGE) following previously reported procedures [12].

2.3. Characterisation of plasmids

Plasmids from isolates Ec001 and Ec002 were extracted using a PureLink™ HiPure Plasmid Filter Midiprep Kit (Invitrogen, Milan, Italy). Purified plasmid DNA was used for preparation of DNA paired-end libraries generated using the Nextera XT DNA Sample Preparation Kit (Illumina Inc., San Diego, CA) and was sequenced using an Illumina MiSeq instrument with 2×300PE protocol (Illumina Inc.). De novo assembly of Illumina reads was performed using SPAdes 3.8 software through the ARIES public Galaxy server (https://w3.iss.it/site/aries/). Contigs were screened for plasmid and resistance gene content using PlasmidFinder and ResFinder tools, respectively, at the Center for Genomic Epidemiology (CGE) server (https://cge.cbs.dtu.dk/services/). Replicon alleles were assigned at the plasmid MLST site (https://pubmlst.org/plasmid/). The order and orientation of contigs with overlapping paired-ends was initially performed following the assembly of two reference plasmids, pCTXM15_EC8 (**KP789020.1**) and FDAARGOS_434 unnamed plasmid 1 (**CP023871.1**), identified in *E. coli* from China and Canada, respectively. The pNDM-5-IT complete sequence was confirmed by the PCR-based gap closure method and Sanger sequencing of the amplicons.

Circular plasmid sequences were annotated at the Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/).

Table 1
Data on patients with NDM-producing *Escherichia coli* and main characteristics of the isolates.

Patient	Isolate	Sample	Carbapenemase	ESBL	MIC (mg/L) [susceptibility category]														Phylogroup	ST/CC
					MEM	ETP	IPM	AMC	CTX	CAZ	FEP	CIP	AMK	GEN	SXT	TGC	COL	FOS	NIT	TZP		
1	Ec001	Urine	NDM-5	TEM-32	64 [R]	>1 [R]	>16 [R]	>8/2 [R]	>4 [R]	>128 [R]	>32 [R]	>2 [R]	≤4 [S]	>4 [R]	>4/76 [R]	0.25 [S]	≤0.5 [S]	≤16 [S]	≤32 [S]	>128/4 [R]	A	ST167/CC10
2	Ec002	Urine	NDM-5	-	>64 [R]	>1 [R]	>16 [R]	>8/2 [R]	>4 [R]	>128 [R]	>32 [R]	>2 [R]	≤4 [S]	>4 [R]	>4/76 [R]	0.25 [S]	≤0.5 [S]	≤16 [S]	64 [S]	128/4 [R]	A	ST167/CC10
2	Ec003	Faeces	NDM-5	-	>64 [R]	>1 [R]	>16 [R]	>8/2 [R]	>4 [R]	>128 [R]	>32 [R]	>2 [R]	≤4 [S]	>4 [R]	>4/76 [R]	0.25 [S]	≤0.5 [S]	≤16 [S]	64 [S]	>128/4 [R]	A	ST167/CC10

ESBL, extended-spectrum β-lactamase; MIC, minimum inhibitory concentration; MEM, meropenem; ETP, ertapenem; IPM, imipenem; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; TGC, tigecycline; COL, colistin; FOS, fosfomycin; NIT, nitrofurantoin; TZP, piperacillin/tazobactam; ST, sequence type; CC, clonal complex; R, resistant; S, susceptible.

Table 2
Plasmid content of NDM-producing *Escherichia coli* isolates.

Isolate	Plasmid	Size (bp)	Replicon content	Resistance genes
Ec001	pNDM-5-IT	99 476	FII (allele 36)	bla_{NDM-5}, *aac(3)-IIa, aadA2, mphA, sul1, tetA, dfrA12*
			FII (allele 31) FIA (allele 4) FIB (allele 1)	
	pX1	38 611	X1	bla_{TEM-32}
	pMG2	5167	Col156	
	pMG1	4773	ColRNAI	
Ec002	pNDM-5-IT	99 476	FII (allele 36)	bla_{NDM-5}, *aac(3)-IIa, aadA2, mphA, sul1, tetA, dfrA12*
			FII (allele 31) FIA (allele 4) FIB (allele 1)	
	pMG2	5167	Col156	
	pMG1	4773	ColRNAI	

3. Results

3.1. Patients and NDM-producing Escherichia coli isolates

The three NDM-producing *E. coli* isolates (Ec001, Ec002 and Ec003) analysed in this study were obtained from two patients aged 60–70 years with urinary tract infection (UTI) admitted to the same hospital (Hospital 'Lorenzo Bonomo', Andria, Puglia Region, Italy) 2 months apart in 2017. Patient 1 was admitted to the neurosurgery unit of the hospital, whilst Patient 2 presented to the outpatient clinic. Ec001 and Ec002 were recovered from the urine cultures of Patients 1 and 2, respectively, whilst Ec003 was detected in a rectal swab sample from Patient 2. No NDM-producing isolate was identified in the faeces of Patient 1. Both patients were treated with fosfomycin although with a different therapeutic scheme (a single 3 g dose for Patient 1 and two 3 g doses 24 h apart for Patient 2) and both recovered. At a check-up 4 months later, Patient 2 was found to be negative for NDM-producing *E. coli* by urine culture, although faecal colonisation persisted. Neither patient reported a recent travel history to any NDM-endemic area. The three isolates (Ec001, Ec002 and Ec003) were sent to the Istituto Superiore di Sanità (Rome, Italy) for further phenotypic and genotypic characterisation.

3.2. Antibiotic susceptibility profile and genotyping of Escherichia coli

All three *E. coli* isolates shared the same resistance profile. As shown in Table 1, the isolates were resistant to meropenem, ertapenem, imipenem, amoxicillin/clavulanic acid, third- and/or fourth-generation cephalosporins, ciprofloxacin, gentamicin, trimethoprim/sulfamethoxazole and piperacillin/tazobactam but remained susceptible to nitrofurantoin, colistin, amikacin, fosfomycin and tigecycline. All three isolates were found to carry the bla_{NDM-5} gene. Results of phenotypic tests were consistent with the type of carbapenemase produced. Ec001 also harboured the bla_{TEM-32} ESBL gene, but the isolates from Patient 2 (Ec002 and Ec003) did not. No other carbapenemase/ESBL gene was detected. All three isolates belonged to phylogenetic group A, clonal complex 10 (CC10) and ST167. The isolates showed an identical profile by PFGE (data not shown).

3.3. Plasmid content of Ec001 and Ec002

Plasmid next-generation sequencing analysis was conducted on the two UTI isolates (Ec001 and Ec002) from Patients 1 and 2, respectively. PlasmidFinder performed on assembled contigs from Ec001 and Ec002 detected the same plasmid content in both isolates, with the exception of an X1 plasmid that was found in Ec001 isolate only (Table 2).

The plasmid carrying bla_{NDM-5} was designated pNDM-5-IT, belonged to the IncF family and also harboured *dfrA12, aadA2, sul1, aac(3)-IIa, mphA* and *tetA*(A) resistance genes. Plasmid pX1_1 carried only the bla_{TEM-32} gene. Two different small plasmids (named pMG1 and pMG2) were also identified matching with the pIGMS32 (**DQ298019**) and pIGJC156 (**NC_009781**) sequences, respectively. These latter encoded the basic rolling circle replication controls and the MobA–L mobilisation systems.

3.4. Features and characteristics of the pNDM-5-IT plasmid

Within the pNDM-5-IT plasmid, the bla_{NDM-5} gene was found in a complex integron, bracketed by two IS*26*, containing an IS*CR1* element and a class 1 integron with the *intI1* gene truncated by one of the IS*26* copies and the *aadA2–dfrA12* resistance gene cassettes (Fig. 1).

pNDM-5-IT showed a peculiar scaffold constituted by four replicons of the IncF type (FIA, FIB and two copies of the FII replicon). Plasmid MLST assigned this plasmid to the FAB formula F36:F31:A4:B1. The FII allele 36 differs by one nucleotide from the FII allele 31 and is not functional since the replication protein *repA* gene was destroyed by the insertion of an IS*1* element.

By BlastN, the best match with pNDM-5-IT (99% nucleotide identity and 81% coverage) was observed with plasmid pCTXM15_EC8 identified in WCHEC13-8 *E. coli* strain ST3835 isolated in China [13]. pCTXM15_EC8 also showed FIA, FIB and two FII replicons, with the highly related FAB formula F36:F36:A4:B1.

Comparative analysis between pNDM-5-IT and pCTXM15_EC8 showed that the scaffold of both plasmids was highly conserved, including the replicons, stabilisation, toxin–antitoxin systems (*sopA, sopB, ccdA, ccdB, pemI, pemK*) and putative virulence factors such as the arginine deaminase (ADI) cluster (*arcA, arcB, arcC* and *arcD* genes) flanked by two inverted repeated IS*66*–IS*1* elements but also the iron/manganese ABC transporter and the aerobactin Iuc system (Fig. 1). The deleted FII replicon was trapped by the element constituted by two inverted repeated IS*66*–IS*1* sequences carrying the ADI cluster. The Tra locus for conjugation was intact in pCTXM15_EC8 but was partial in pNDM-5-IT (only the *traX* and *finO* genes were detected), indicating that pNDM-5-IT is not able to promote self-conjugation. pCTXM15_EC8 and pNDM-5-IT differed for the resistance determinant content: pNDM-5-IT carried the *dfrA12–aadA2–*IS*CR1–*bla_{NDM-5} complex integron and the macrolide (*mphA–mphR*) and gentamicin [*aac(3)-IIa*] resistance genes, whilst in the same plasmid region pCTXM15_EC8 showed the IS*Ecp1–*$bla_{CTX-M-15}$ module and the *aacA4,* bla_{OXA-1} and *cat* resistance genes. Plasmid pNDM-5-IT also matched (99% nucleotide identity, 54% coverage) with an NDM-5-positive plasmid identified in an *E. coli* strain isolated in Canada (FDAARGOS_434).

pFDAARGOS_434 plasmid carried the FII and FIA replicons with a FAB formula F36:A4:B-. Comparison among plasmids showed that the region comprising the bla_{NDM-5}-containing integron and the *mphA–mphR* genes was identical in pFDAARGOS_434 and pNDM-5-IT plasmids. The region encoding the FIB, the ABC-aerobactin Iuc system identified in pCTX15_EC8 and pNDM-5-IT was missing in pFDAARGOS_434, that showed, in this place, a different ABC/iron transport system (Fig. 1). Finally, pFDAARGOS_434 lacked the ADI cluster with the second deleted copy of FII.

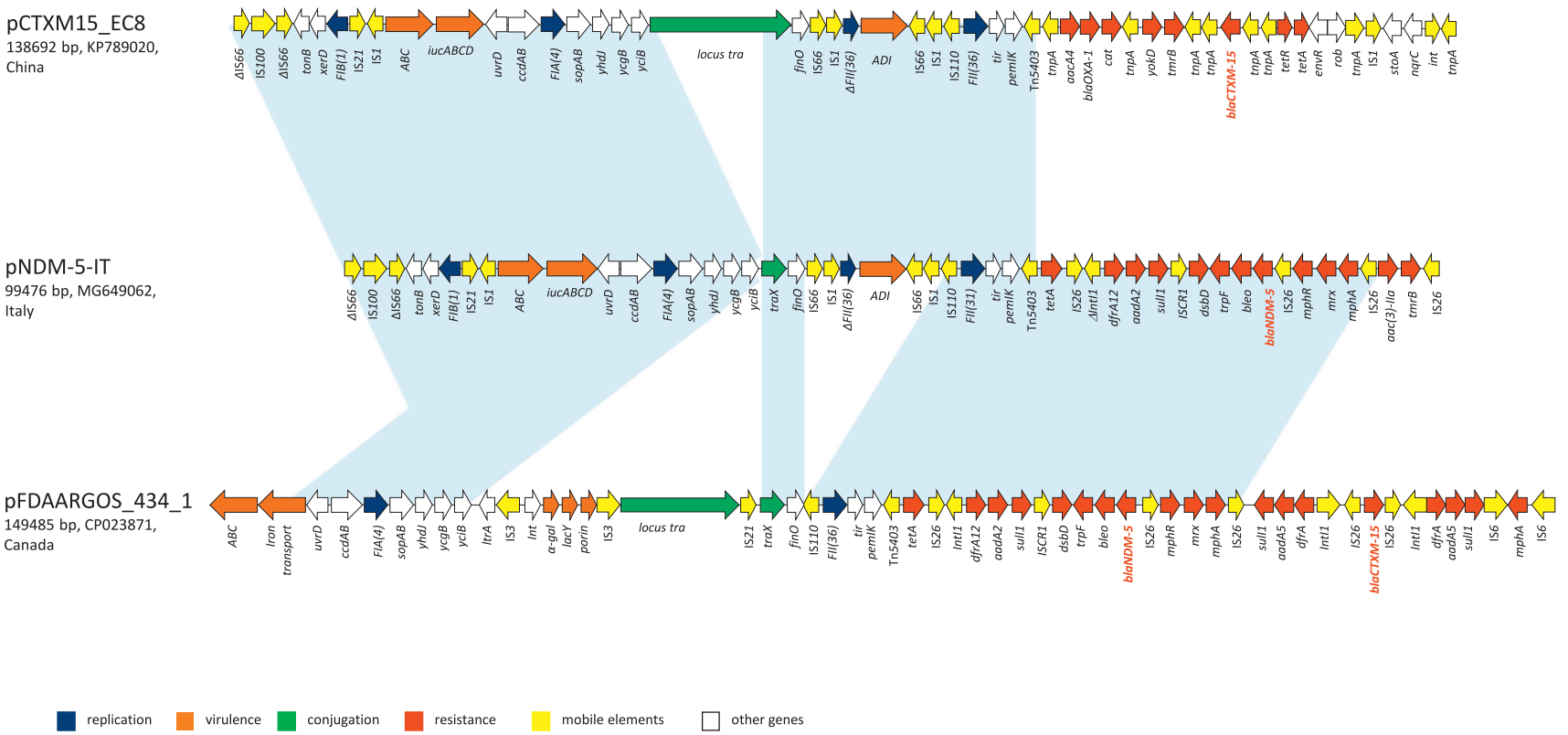

Fig. 1. Major structural features of plasmid pNDM-5-IT in comparison with pCTXM15_EC8 and pFDAARGOS_434_1. Predicted coding sequences are indicated by coloured arrows oriented in the direction of transcription of each respective gene: red arrows, resistance genes; yellow arrows, transposon-related genes and insertion sequences; blue arrows, replicons; white arrows, other genes of the plasmid scaffold; brown arrows, clusters encoding putative virulence factors or other virulence genes. The transfer locus *tra* and associated *trb* genes are indicated by a continuous green arrow.

4. Discussion

Here we report on the first detection of *E. coli* ST167 isolates carrying an NDM-5 carbapenemase on a novel IncF plasmid in Italy. Enterobacteriaceae carrying NDM enzymes have rarely been observed in our country [4,5,14,15]. According to a recent survey on carbapenemase-producing Enterobacteriaceae in Europe (EuSCAPE), an irrelevant proportion of NDM-positive *K. pneumoniae* and/or *E. coli* isolates (0.5% and 0%, respectively) was observed among Italian isolates [3].

The three NDM-5-producing isolates described in this study (i) displayed overlapping resistance phenotypes, (ii) belonged to the same ST167 clone, (iii) were identical by PFGE and (iv) harboured the same plasmid content, suggesting they were actually the same strain. Transmission of the strain from one patient to the other could be hypothesised but we were unable to document a direct epidemiological link. In the literature, ST167 was previously found associated with NDM-5 mainly in China where it was recognised as an epidemic clone of significant public-health concern carrying plasmids associated with antimicrobial resistance [16–18]. In Europe, the ST167 clone has been sporadically detected. In Italy, it was found to occur very rarely [10] or not at all [19]. As observed in China, the ST167 isolates detected in this study were highly resistant to several different classes of antimicrobial agents [16,18]. Accordingly, besides the $bla_{\text{NDM-5}}$ gene, plasmid pNDM-5-IT harboured other resistance determinants conferring resistance to trimethoprim, sulfonamides, aminoglycosides, macrolides and tetracycline. However, it is noteworthy that the ST167 isolates remained susceptible to old antimicrobial agents such as nitrofurantoin and fosfomycin, the latter successfully used in the treatment of both of patients.

In Italy, the only other cases of infection due to an NDM-5-carrying *E. coli* were found to be associated with ST405 [5]. The plasmid in the ST405 isolate belonged to the IncFII group but the scaffold was highly different from that identified herein in ST167. In fact, the pNDM-5-IT plasmid had a scaffold characterised by the double FII feature. Looking at the complete plasmid sequences available in GenBank, double alleles (either F36:F31 or F36:F36) were present in 17 plasmids, of which 15 also showed the FIA allele A4 (data not shown). Most of these plasmids were associated with the presence of the $bla_{\text{CTX-M-15}}$ gene (9/17) but not with the $bla_{\text{NDM-5}}$ gene. It is therefore plausible that either the $bla_{\text{NDM-5}}$ or the $bla_{\text{CTX-M-15}}$ gene had independent occasions to join this type of IncF plasmid that is very frequent in *E. coli*. Of note, in this study we noted the association of the double FII feature with the ADI cluster. The ADI pathway converting L-arginine into L-ornithine is the most widespread anaerobic route for arginine degradation contributing to survival of bacteria in acidic environments [20]. The ADI cluster is widely present in different bacterial species and genera, but it has not been previously described linked to a plasmid. This genetic determinant could be considered a plasmid-mediated virulence factor in addition to the aerobactin system for iron uptake also carried by the pNDM-5-IT plasmid.

In conclusion, an important feature of NDM-producers is their ability to spread rapidly both in healthcare and community settings [21]. Considering that *E. coli* is the leading cause of UTI, the occurrence of the NDM-5 enzyme in the ST167 clone that, although rare so far in Italy, is frequent in NDM-endemic regions such as China, is of concern. Moreover, the NDM-5-producing ST167 strain characterised herein was found to carry the $bla_{\text{NDM-5}}$ gene on a plasmid possessing both virulence and resistance features that in combination may facilitate its further spread. Overall, this study underlines the importance of surveillance and investigation of carbapenem-resistant clones and associated genes and plasmids.

Sequence accession numbers

Plasmid nucleotide sequences have been deposited in GenBank with the following accession nos.: **MG649062** (pNDM-5-IT); **MG649063** (pX1); **MG649064** (pMG1); and **MG649065** (pMG2).

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Competing interests

None declared.

Ethical approval

Not required.

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