

Detection and characterization of pCT-like plasmid vectors for *bla*_{CTX-M-14} in *Escherichia coli* isolates from humans, turkeys and cattle in England and Wales

M. O. Stokes^{1,2*}, J. L. Cottell³, L. J. V. Piddock³, G. Wu⁴, M. Wootton⁵, D. J. Mevius⁶, L. P. Randall¹, C. J. Teale¹, M. D. Fielder² and N. G. Coldham¹

¹Department of Bacteriology, Animal Health and Veterinary Laboratories Agency, Addlestone, UK; ²Faculty of Science, Engineering and Computing, Kingston University, Kingston, UK; ³School of Immunity and Infection, The College of Medicine and Dental Sciences, University of Birmingham, Birmingham, UK; ⁴Center for Epidemiology and Risk Analysis (CERA), Animal Health and Veterinary Laboratories Agency, Addlestone, UK; ⁵Public Health Wales, University Hospital of Wales, Cardiff, UK; ⁶Department of Bacteriology and TSEs, Central Veterinary Institute of Wageningen, Lelystad, The Netherlands

*Corresponding author. Tel: +44-1932-357246; Fax: +44-1932-347706; E-mail: m.stokes@vla.defra.gsi.gov.uk

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Objectives: To detect and characterize *Escherichia coli* strains and pCT-like plasmids implicated in the dissemination of the CTX-M-14 gene in animals and humans, in England and Wales.

Methods: UK CTX-M-14-producing *E. coli* ($n=70$) from cattle ($n=33$), turkeys ($n=9$), sheep ($n=2$) and humans ($n=26$) were screened using multiplex PCR for the detection of a previously characterized plasmid, pCT. Isolates found to be carrying two or more pCT genetic markers were further analysed using PFGE. Their antimicrobial-resistance genes and virulence genes were also determined. These plasmids were transferred to *Salmonella enterica* serotype Typhimurium 26R and further examined for incompatibility type, genetic environment of the *bla*_{CTX-M-14} gene, size, restriction fragment length polymorphism (RFLP) and *nikB* sequence.

Results: The 25 *E. coli* isolates carrying pCT genetic markers generated 19 different PFGE profiles, and 23 isolates had different virulence and antimicrobial-resistance gene patterns. One isolate from cattle was a verotoxinogenic *E. coli* ('VTEC'); the rest were commensal or extra-intestinal pathogenic *E. coli*. pCT-like plasmids with similar molecular characteristics (size, replicon type, RFLP pattern, pCT markers and genetic environment of the *bla*_{CTX-M-14} gene) were detected in 21/25 of the field isolates, which comprised those from cattle ($n=9$), turkeys ($n=8$) and humans ($n=4$). All pCT-like plasmids were conjugative, and most were IncK ($n=21$) and had the same local genetic environment flanking the *bla*_{CTX-M-14} gene ($n=23$). RFLP analysis demonstrated $\geq 75\%$ similarity among most plasmids ($n=22$).

Conclusions: pCT-like plasmids were common vectors for horizontal dissemination of 30% of the *bla*_{CTX-M-14} genes to different *E. coli* isolates from humans, cattle and turkeys.

Keywords: plasmids, CTX-M-14, IncK, pCT, ESBLs, antimicrobial resistance

Introduction

Third- and fourth-generation cephalosporins are commonly used for treating infections in humans and animals caused by Enterobacteriaceae. However, their efficacy is being compromised by extended-spectrum β -lactamases (ESBLs) and cephamycinases (AmpC) produced by those bacteria. Since the early 1990s *bla*_{CTX-M} genes have become the most common plasmid-borne ESBL genes, aiding their horizontal transmission among different *Escherichia coli* strains and other species of Enterobacteriaceae.^{1–3} Plasmids of numerous incompatibility groups encoding various *bla*_{CTX-M} genes have been found in bacterial species from human and

veterinary sources across the globe.^{1,4,5} The first UK veterinary CTX-M isolate was reported in 2004.⁶ This *E. coli* was isolated from cattle and was found to harbour a 93.6 kb IncK plasmid that encoded *bla*_{CTX-M-14}. This plasmid has been designated 'pCT' and sequenced, which has allowed the design of a multiplex PCR assay for the detection of similar plasmids.^{7,8} Whilst the *bla*_{CTX-M-14} gene remains uncommon in UK human isolates, it has been found to be prevalent in *E. coli* from cattle and turkeys.^{9,10} In Spain, France and Asia the *bla*_{CTX-M-14} gene is frequently detected in human *E. coli* isolates.^{11–13}

The aim of this study was to investigate the mode of dissemination of *bla*_{CTX-M-14} in bacteria isolated from cattle, turkeys and

humans in England and Wales, by molecular typing of CTX-M-14-producing *E. coli* and their plasmids.

Materials and methods

Bacterial strains

All CTX-M-14-producing *E. coli* isolates of veterinary origin in the Animal Health and Veterinary Laboratories Agency (AHVLA) culture collection ($n=44$) were selected and grown on Chromagar CTX or Luria–Bertani minus glucose (LB–G) agar plates with 2 mg/L cefotaxime at 37°C. The isolates from cattle (C, $n=33$) and sheep (S, $n=2$) were obtained through routine surveillance or diagnostic submissions, while those from turkeys (T, $n=9$) were obtained during a study on the prevalence of CTX-M in poultry;¹⁰ with the exception of C8 and C9, all were from different farms and they consisted of eight faecal isolates and one caecum isolate from healthy animals.¹⁰ All human (H) CTX-M-14 isolates were isolated from urine samples (except one unknown) obtained from individual patients from Wales ($n=18$) and England ($n=8$) through hospital or community submissions. Field isolates producing CTX-M-1 isolated from cattle ($n=7$), turkeys ($n=5$) and chickens ($n=15$), CTX-M-3 from cattle ($n=1$) and chicken ($n=1$) and CTX-M-15 from cattle ($n=8$) were grown as described above, and used in this study to compare the specific association of pCT-like plasmids for the *bla*_{CTX-M-14} gene.

Analysis of *E. coli* isolates

The clonal relatedness of CTX-M-14 *E. coli* field isolates was examined by XbaI PFGE and antimicrobial and virulence DNA arrays. PFGE was carried out following the CDC PulseNet protocol and the results were analysed using the Dice coefficient (Bionumerics 5.10, Applied Maths), with a cut-off of 85% similarity used to define distinct clusters.¹⁴ DNA array (Alere Inc.) analysis was performed on *E. coli* field isolates and *Salmonella enterica* serotype Typhimurium 26R transconjugants, as previously described.¹⁵ This version of the array has 153 probes for antimicrobial resistance and 120 for virulence genes.¹⁶ GeneSpring (Agilent Technologies) was used for cluster analysis of the gene array data.

PCR analysis of plasmids

Suitable positive and negative controls were included for all PCRs. The *bla*_{CTX-M} sequence type was confirmed by PCR, using primers described by Batchelor et al.,¹⁷ and DNA sequence analysis. Investigation of the *bla*_{CTX-M-14} genetic context and the presence of the insertion sequence *ISEcp1*, the pCT multiplex PCR testing for sigma factor, shufflon recombinase, *pilN* and pCT008-009 and the *nikB* sequencing for transconjugants were all performed as described by Cottell et al.⁷ (see Table 1). Inck replicon typing was carried out as described by Carattoli et al.,¹⁸ but with a new forward primer 5'-CAGGATCCGGGAAGTCAGAAAC-3', designed from the pCT sequence (FN868832.1).

Molecular characterization of plasmids

Veterinary and human isolates containing *bla*_{CTX-M-14} plasmids with at least two pCT genetic markers were transferred by conjugation to rifampicin-resistant *Salmonella* Typhimurium 26R, enabling further characterization of plasmids in an identical host background, as described previously by Randall et al.¹⁰ Sizing of the plasmids was carried out using S1 nuclease PFGE, with pCT, low-range (2030–194 000 bp) and mid-range (15 000–242 500 bp) PFGE molecular marker ladders (NEB) as size calibration standards.¹⁹ Restriction fragment length polymorphism (RFLP) analysis of the plasmids was performed as previously described.⁸

Results

Molecular analysis of veterinary and human *E. coli* isolates

A total of 25 (35.7%, $n=7$ from humans, $n=9$ from cattle and $n=9$ from turkeys) of the 70 CTX-M-14-producing *E. coli* field isolates were found to encode two or more pCT genetic markers (sigma factor, shufflon recombinase, *pilN* and pCT008-009) (Table 1). These isolates were selected for further study. Of the non-CTX-M-14-producing veterinary isolates ($n=37$), two cattle CTX-M-1-producing isolates had sigma factor and shufflon recombinase pCT genetic markers on IncF plasmids, and three chicken CTX-M-1-producing isolates had the shufflon recombinase pCT genetic markers on IncI1 plasmids. Additionally, two of the CTX-M-15-producing isolates from cattle had two pCT genetic markers (sigma factor with *pilN*, and sigma factor with shufflon recombinase; results not shown).

The XbaI PFGE for the 25 CTX-M-14-producing *E. coli* isolates with pCT markers identified 19 unique clusters at 85% similarity, and identified H5 and H6 as clones (Figure 1). With the exceptions of the human H5 and H6 clones and T5 and T7 from turkeys, all isolates had different genes for antibiotic resistance (Figure S1a, available as Supplementary data at JAC Online) and virulence (Figure S1b, available as Supplementary data at JAC Online) as determined by microarrays; assigned arrays and PFGE types are stated in Table 1. This supported the PFGE profile and demonstrated that the *E. coli* isolates were clearly unrelated. Analysis of virulence genes (Figure S1b) identified a verotoxigenic *E. coli* ('VTEC') strain (C7) harbouring *stx1A*, *eae*, *hlyA* and type III secretion systems genes, such as *nleA*. Other isolates contained genes that were found among extra-intestinal *E. coli* (ExPEC) or commensal isolates. The serum-resistance gene (*iss*) and the gene for long polar fimbriae (*lpf*) were found in a high proportion of isolates (80% and 56%, respectively). Virulence genes including *pic*, *vat*, *tsh*, *nfaE*, *sat* and *pfrB*, which are typically found in ExPEC, were identified among this group of strains. Genes that are often associated with ExPEC, such as those for iron utilization, *iroN* and *ireA*, and those for microcin production, *celb*, *mchBCF*, *mcmA* and *cma*, were found in various strains in this study (Figure S1b).

Molecular analysis of plasmids

All *bla*_{CTX-M-14} plasmids harbouring two or more pCT genetic markers were conjugated into *Salmonella* Typhimurium 26R. The pCT PCR showed that 19/25 (76%; human $n=4$, cattle $n=8$ and turkey $n=7$) encoded all four pCT genetic markers, three had three genetic markers (12%; one each from human, cattle and turkey), two human isolates had two genetic markers (8%) and the transconjugant of T4 had one marker (Table 1). However, pCT markers were in the field isolates harbouring pMSC2, which contained sigma factor, pMST4 had shufflon recombinase, *pilN*, and pCT008-009 and pMST6 had sigma factor.

With the exception of pMSH5, pMSH6, pMSH7 and pMST4, all of the plasmids ($n=21/25$) belonged to the IncK replicon type (Table 1). All of the *bla*_{CTX-M-14} plasmids ($n=23/25$), apart from pMSH5 and pMSH6, had the same *ISEcp1*-*bla*_{CTX-M-14}-*pseudogene R* genetic environment (Table 1). Sequence analysis of *nikB* showed that most of the plasmids ($n=23/25$) had the same

Table 1. The 25 *E. coli* isolates with two or more pCT genetic markers used in this study

Plasmid ID	Host species	Location	Year of isolation	Sample	PFGE, AMR and virulence types	<i>E. coli</i> field isolate	<i>Salmonella</i> Typhimurium 26R transconjugant	<i>bla</i> _{CTX-M-14}	ISEcp1- <i>bla</i> _{CTX-M-14} - <i>pseudogene R</i>	Plasmid size (kb)	IncK replicon type	<i>nikB</i> sequence	
						PCR multiplex	PCR multiplex						
pMSC1	cattle	England	2010	faeces	1, 1, 1	S, R, N, P	S, R, N, P	+	+	90	+	+	
pMSC2	cattle	Wales	2010	faeces	2, 2, 2	S, R, N, P	R, N, P	+	+	85	+	+	
pMSC3	cattle	Wales	2010	faeces	3, 3, 3	S, R, N, P	S, R, N, P	+	+	110	+	+	
pMSC4	cattle	England	2010	faeces	4, 4, 4	S, R, N, P	S, R, N, P	+	+	90	+	+	
pMSC5	cattle	England	2010	faeces	1, 5, 1	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSC6	cattle	Wales	2006	faeces	5, 6, 5	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSC7	cattle	England	2008	faeces	6, 7, 6	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSC8	cattle	Wales	2006	caecum	7, 8, 7	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSC9	cattle	Wales	2006	faeces	7, 9, 8	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSH1	human	Wales	2007	urine	8, 10, 9	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSH2	human	Wales	2007	urine	9, 11, 10	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSH3	human	Wales	2007	urine	10, 12, 11	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMSH4	human	Wales	2007	unknown	11, 13, 12	S, R, N, P	S, R, N, P	+	+	105	+	+	
pMSH5	human	Wales	2007	urine	12, 14, 13	S, N	S, N	+	-	100	-	+	(9)
pMSH6	human	Wales	2007	urine	12, 14, 13	S, N	S, N	+	-	100	-	+	(9)
pMSH7	human	England	2009	urine	13, 15, 14	S, R, N	S, R, N	+	+	100	-	+	
pMST1	turkey	England	2006	faeces	14, 16, 15	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMST2	turkey	England	2006	faeces	15, 17, 16	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMST3	turkey	England	2006	faeces	16, 18, 17	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMST4	turkey	England	2006	faeces	16, 4, 17	S, R, N, P	S	+	+	ND	-	+	(FI)
pMST5	turkey	England	2006	faeces	17, 12, 18	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMST6	turkey	England	2006	faeces	18, 19, -19	S, R, N, P	R, N, P	+	+	95	+	+	
pMST7	turkey	England	2006	faeces	17, 12, 18	S, R, N, P	S, R, N, P	+	+	100	+	+	
pMST8	turkey	England	2006	faeces	19, 20, 20	S, R, N, P	S, R, N, P	+	+	95	+	+	
pMST9	turkey	England	2006	faeces	19, 21, 21	S, R, N, P	S, R, N, P	+	+	95	+	+	

FI, field isolate used for *nikB* sequencing; unknown, not recorded.

pCT markers: S, sigma factor (1289 bp); R, shufflon recombinase (945 bp); N, *pilN* (627 bp); and P, pCT008-009 (428 bp).

ISEcp1-*bla*_{CTX-M-14}-*pseudogene R* is the genetic environment surrounding *bla*_{CTX-M-14}.

The accession number for the pCT plasmid is FN868832.1.

DH5 α pCT was used as a positive control, and *Salmonella* Typhimurium 26R without plasmid pCT was used as a negative control.

Assigned PFGE, antimicrobial resistance (AMR) and virulence types are all indicated numerically; for PFGE a cut-off at 85% similarity was applied, and for AMR and virulence genes profiles, a difference of one gene was used to define new groups.

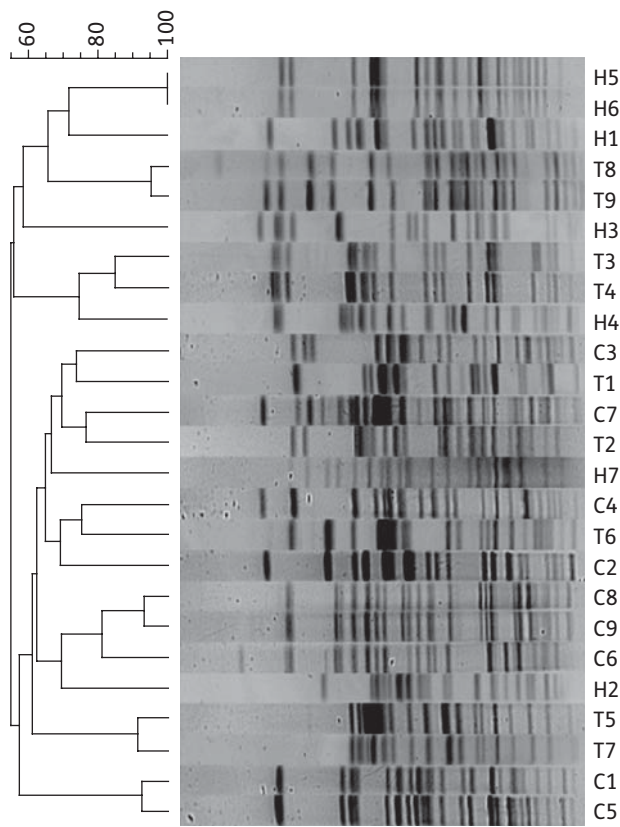


Figure 1. XbaI PFGE of the 25 *E. coli* isolates containing *bla*_{CTX-M-14} pCT-like plasmids isolated from humans, cattle and turkeys. *Salmonella* Braenderup was used as the control to align the banding profiles. Similarities in banding patterns were calculated using the Dice coefficient (Bionumerics 5.10). C, cattle isolates; H, human isolates; T, turkey isolates.

sequence as pCT (this includes the sequence from the T4 field isolate). Variations were only seen in plasmids pMSH5 and pMSH6, which had nine nucleotide substitutions relative to pCT: nucleotides 81 (T→C), 90 (C→T), 96 (T→G), 129 (A→C), 130 (T→G), 216 (A→G), 222 (C→G), 231 (T→C) and 240 (A→G).

RFLP analysis of the plasmids in the transconjugants with restriction enzymes PstI (Figure S2, available as Supplementary data at JAC Online) and EcoRI (results not shown) revealed that 22/25 (88%) of the plasmids had 75%–85% similarity in banding patterns, while the clonal plasmids pMSH5 and pMSH6 had 65% similarity to the other pCT-like plasmids. There was also a small cluster at the 70% similarity level following EcoRI digestion, which included plasmids pMSC2, pMSC3 and pMSH4. S1 nuclease sizing showed that 15 plasmids were of similar size to pCT (93.6 kb, 95 kb by S1 nuclease sizing), 6 were larger (100–110 kb) and 3 were smaller (85–90 kb) (Table 1). Differences in RFLP patterns were consistent with the varying sizes of plasmids. The T4 transconjugant had no detectable plasmid or IncK or *nikB* sequence, but had the *bla*_{CTX-M-14} gene in the same genetic environment and the sigma factor genetic marker. Aminoglycoside (*aac6*) resistance gene was identified on pMSH5 and pMSH6 by DNA array. The results of the molecular typing data demonstrated that 21/25 *bla*_{CTX-M-14} plasmids

shared a high level of similarity to each other and to pCT; this comprised 30% of all CTX-M-14 *E. coli* tested.

Discussion

Until now, *bla*_{CTX-M-14} IncK pCT plasmids from the UK have only been reported in cattle. In this study, *bla*_{CTX-M-14} IncK pCT-like plasmids were identified in 21/70 (30%) of CTX-M-14 *E. coli* isolates from humans, cattle and turkeys, of which 17 of the field isolates were unrelated to each other based on molecular typing by PFGE, antimicrobial-resistance and virulence microarrays. Plasmids were defined as being pCT-like by the presence of three or more pCT markers: the *ISEcp1-bla*_{CTX-M-14}-*pseudogene R* genetic environment, *nikB* sequence and the IncK replicon type. Although there were some variations among the pCT-like plasmids in terms of RFLP patterns, size or genetic markers, they shared a similar genetic backbone with pCT. The variations in pCT-like plasmids are likely to have occurred through mutation and recombination. Some markers identified in the field isolates but not in the transconjugants could be located on the chromosome or on other plasmids. Also, since no plasmid and only genes for *ISEcp1-bla*_{CTX-M-14}-*pseudogene R* and sigma factor were detected in the transconjugant of T4, it is likely that part of the plasmid from the T4 field isolate was integrated into the chromosome of the transconjugant, as all of the pCT markers, *nikB* and a plasmid of similar size were detected in the field isolate. The absence of IncK in pMSH5, pMSH6 and pMSH7 may be the result of the plasmids belonging to an alternative replicon type, and along with pMST4, these were not described as pCT-like plasmids. Only 10.8% ($n=4/37$) of non-CTX-M-14-producing isolates (CTX-M-1, CTX-M-3 and CTX-M-15) had at least two of the pCT genetic markers, none of which was IncK, demonstrating a strong association of *bla*_{CTX-M-14} with pCT and IncK ($n=21/25$). Individual pCT markers are not IncK-specific and can be found in other plasmids, but these markers are designed to work in combination for detection of pCT-like plasmids.

All pCT-like plasmids in this study were successfully conjugated to a *Salmonella* recipient strain, as was reported previously for pCT-like plasmids from the outbreak farm.⁸ Therefore, conjugation was the most likely mechanism for the spread of pCT-like plasmids to diverse *E. coli* strains recovered from humans, cattle and turkeys. This demonstrates that pCT-like plasmids are important vectors for the dissemination of the *bla*_{CTX-M-14} gene. Similar reports from Korea show that IncF *bla*_{CTX-M-14} plasmids have a role in disseminating the *bla*_{CTX-M-14} gene between *E. coli* that have no major clonal relationship based on either PFGE or multilocus sequence typing.¹³

This mechanism for CTX-M-14 dissemination by pCT-like plasmids is in contrast to that for the spread of CTX-M-15, where the pandemic *E. coli* clone O25:ST131 plays a dominant role.^{20–22} This successful *E. coli* clone has been found to disseminate *bla*_{CTX-M-15} and other *bla*_{CTX-M} genes on various plasmids in the UK and across Europe.^{21–23} In addition, mobile elements, such as integrons and transposons, also play important roles in mobilizing resistance genes onto different plasmids and chromosomes.^{24–26}

The horizontal transmission of plasmids between unrelated *E. coli* and *Salmonella* in animals has been shown previously.^{8,27} Several reports have commented on the transmission of plasmids

between human and veterinary isolates. These include IncN bla_{CTX-M-1} plasmids in unrelated *E. coli* isolates from humans and pigs and the IncI1 bla_{CTX-M-1}, IncHI2 bla_{CTX-M-2} and IncHI2 bla_{CTX-M-9} plasmids in *E. coli* and *Salmonella* isolates from humans and poultry across Europe.^{28–31}

The detection of similar pCT-like plasmids in human and veterinary isolates from England and Wales suggests that these plasmids are effective vectors for the dissemination of bla_{CTX-M} among different animal host species that may be linked by the food chain or the environment. Food has previously been found to be contaminated with quinolone-resistant ESBL bla_{CTX-M-14} *E. coli* and those carrying bla_{CTX-M-1} IncI1 plasmids that were also found in broilers across Europe.^{29,32,33} Several routes exist for the transmission of bla_{CTX-M} genes from humans to animals, and these include land flooded with sewage-contaminated water and various wildlife vectors, including rats and gulls.^{34–36} The presence of the pCT-like plasmids in over 45% of bla_{CTX-M-14}-producing animal isolates examined suggests a reservoir of these vectors in food-producing animals in the UK. Such plasmids may be maintained through the prophylactic and therapeutic use of third- and fourth-generation cephalosporins in veterinary medicine.^{28,37} Further work is needed to identify the contribution of animal reservoirs to the dissemination of bla_{CTX-M-14} IncK pCT-like plasmids through the food chain to humans. In conclusion, pCT-like plasmids were shown to be important vectors for the horizontal dissemination of the bla_{CTX-M-14} gene to clonally unrelated *E. coli* isolates from humans, cattle and turkeys.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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