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Dissemination of pHK01-like incompatibility group IncFII plasmids encoding CTX-M-14 in *Escherichia coli* from human and animal sources, 2002-2010

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Abstract

Few studies have compared CTX-M encoding plasmids identified in different ecological sources. This study aimed to analyze and compare the molecular epidemiology of plasmids encoding CTX-M-14 among strains from humans and animals. The CTX-M-14 encoding plasmids in 160 *E. coli* isolates from animal faecal (14 pigs, 16 chicken, 12 cats, 8 cattle, 5 dogs and 3 rodents), human faecal (45 adults and 20 children) and human urine (37 adults) sources in 2002-2010 were characterized by molecular methods. The replicon types of the CTX-M-14 encoding plasmids were IncFII (*n*=61), I1-Iγ (*n*=24), other F types (*n*=23), B/O (*n*=10), K (*n*=6), N (n=3), A/C (n=1), HI1 (n=1), HI2 (n=1) and nontypeable (*n*=30). The genetic environment, IS*Ecp1* - $bla_{\text{CTX-M-14}}$ - IS*903* was found in 89.7% (52/58), 87.7% (57/65) and 86.5% (32/37) of the animal faecal, human faecal and human urine isolates, respectively. Subtyping of the 61 IncFII incompatibility group plasmids by replicon sequence typing, plasmid PCR-restriction fragment length polymorphism and marker genes (*yac*, *malB*, *eitA/eitC* and *parB/A*) profiles showed that 31% (18/58), 30.6% (20/65) and 37.8% (14/37) of the plasmids originating from animal faecal, human faecal and human urine isolates, respectively, were pHK01-like. These 52 pHK01-like plasmids originated from diverse human (20 faecal isolates from 2002, 2007-2008, 14 urinary isolates from 2004) and animal (all faecal, 1 cattle, 1 chicken, 5 pigs, 9 cats, 1 dogs, 1 rodents from 2008-2010) sources. In conclusion, this study highlights the importance of the IncFII group, pHK01-like plasmids in the dissemination of CTX-M-14 among isolates from diverse sources.

Key words: pets, pigs, chicken, plasmid, extended-spectrum beta-lactamases

Introduction

Asia is one of the epicentres of antimicrobial resistance (Hawkey, 2008; Ho et al., 2011c; Ho et al., 2011a). In this part of the world, the CTX-M type extended-spectrum β-lactamases (ESBLs) have spread extensively among bacteria in human, animals and the environment (Hawkey, 2008; Ho et al., 2011a). Many studies have reported the overwhelming dominance of the CTX-M-14 allele among clinical isolates of *Escherichia coli* and *Klebsiella* spp in mainland China, Hong Kong and South Korea (Hawkey, 2008; Ho et al., 2007; Naseer and Sundsfjord, 2011). In general, bacterial strains producing CTX-M-14 are genetically diverse (Ho et al., 2007; Lo et al., 2010; Valverde et al., 2009). Hence, the dissemination of CTX-M-14 has been attributed to conjugative plasmids and other mobile genetic elements rather than clonal expansion (Naseer and Sundsfjord, 2011).

In Spain, the spread of CTX-M-14 from 2000 to 2005 was largely due to pRYC105-like plasmids of the IncK incompatibility group disseminated among diverse *E. coli* lineages (Valverde et al., 2009). It has further been shown that pRYC105 is related to the pCT plasmid that has been found in CTX-M-14 producing strains from the United Kingdom, mainland China and Australia (Cottell et al., 2011). Recently, we sequenced the IncFII incompatibility group plasmid, pHK01 encoding CTX-M-14 and showed that it has disseminated widely among *E. coli* isolates causing community-acquired urinary tract infections in women (Ho et al., 2011b). Plasmids closely related to pHK01 have also been identified among *Klebsiella* strains from mainland China (Yi et al., 2010). Besides plasmids, the acquisition and horizontal transmission of $bla_{\text{CTX-M}}$ genes have been associated with insertion sequences (ISs), putative transposons and class 1 integrons (Canton and Coque, 2006). These elements may have mobilized the $bla_{\text{CTX-M}}$ gene from its progenitor and may drive the expression of the β-lactamase (Canton and Coque, 2006). In addition, these elements might accumulate additional resistance genes to the $bla_{\text{CTX-M}}$ flanking regions (Canton and Coque, 2006). It has

also been suggested that IS*Ecp*, *bla*$_{\text{CTX-M}}$ and IS*903* form a putative transposon and this block of genes could disseminate by transposition (Poirel et al., 2005).

Although the roles of plasmids and other genetic elements are recognized to be important in the dissemination of the CTX-M genes, few studies have compared the distribution of these mobile genetic elements among strains from humans and animals and their inter-relationship (Garcia-Fernandez et al., 2008). In this study, we investigated the molecular epidemiology of plasmids encoding CTX-M-14 and their *bla*$_{\text{CTX-M-14}}$ genetic environment for a collection of *E. coli* isolates collected from diverse human and animal sources between 2002 and 2010.

Methods

Bacterial strains

One hundred and sixty CTX-M-14 producing *E. coli* isolates were studied. The strains were chosen to provide representation from different time periods and animal and human sources. All viable CTX-M-14 producing *E. coli* isolates recovered from four regional antimicrobial resistance surveillances during 2002-2010 were eligible for inclusion (Ho et al., 2007; Ho et al., 2008; Ho et al., 2011a; Lo et al., 2010). These included 37 isolates from female outpatients with urinary tract infections, 65 human faecal isolates (45 adults and 20 children) and 58 faecal isolates from animals (14 pigs, 16 chicken, 12 cats, 8 cattle, 5 dogs and 3 rodents) (Ho et al., 2007; Ho et al., 2008; Ho et al., 2011b; Ho et al., 2011a). The human urine isolates were recovered in 2004 from female outpatients with urinary tract infections (age range, 27 –80 years) and all CTX-M-14 isolates in the collection were included (Ho et al., 2007). The 65 human faecal isolates include all of 62 CTX-M-14 isolates identified in a study that examined faecal carriage in hospitalized children and their household contacts in October 2007-September 2008 (Lo et al., 2010) and three (out of seven CTX-M-14 isolates

found) isolates from a study that assessed carriage in non-hospitalized individuals in 2002 (Ho et al., 2008). The remaining four isolates obtained in the 2002 faecal study were lost during storage and therefore not included. All the animal isolates were obtained during September 2008-August 2010 as part of an ongoing antimicrobial resistance by trained staff in two government departments (Ho et al., 2011a). For cattle and pigs, rectal swabs were obtained from fresh carcasses at a centralized slaughterhouse in Hong Kong (Ho et al., 2011a). Chicken samples were obtained while the animals were temporarily held for inspection before sale at wet markets in Hong Kong. All the cattle were imported from mainland China. The pigs and chicken included animals produced at local farms and those imported from mainland China. Samples from the stray dogs, stray cats and rodents were collected by trained staff at governmental animal management centres. These animals were captured from urban areas from all over Hong Kong. The animals were sampled in batches: chicken (20 animals per batch), cattle (10 animals per batch), pigs (2 to 7 animals per batch), stray cats (1-10 animals batch), stray dogs (1-10 animals per batch) and urban rodents (2 to 23 animals per batch). In total, 2106 animals from 298 batches were tested. In 179 batches, at least one animal was found to carry ESBL-producing *E. coli*. A subset of 132 isolates was chosen randomly according to the collection period and batch number for CTX-M PCR and sequencing. The isolates were selected randomly according to the collection period and batch number. This subset included isolates recovered from 84 batches and covered the entire specimen collection periods. Sequencing showed that 58 of the 132 isolates had CTX-M-14. All the 58 CXT-M-14 producing isolates were included (Ho et al., 2011a).

Susceptibility testing, conjugation and replicon typing

Antimicrobial susceptibility to amoxicillin-clavulanic acid, amikacin, cefotaxime, ceftazidime, ciprofloxacin, chloramphenicol, cotrimoxazole, gentamicin, nalidixic acid,

119 nitrofurantoin, tetracycline was tested by the disc diffusion method (Clinical and Laboratory
120 Standards Institute, 2011). ESBL production was determined by the double-disc synergy test
121 using cefotaxime, amoxicillin-clavulanic acid and ceftazidime at inter-disc distances (centre
122 to centre) of 20 and 25 mm (Ho et al., 2000). Conjugation was carried out in filters with *E.*
123 *coli* J53Az^{r} as the recipient. Donor and recipient cells were mixed at 1:10 ratio.
124 Transconjugants were selected on trypticase soy agar plates containing sodium azide (150
125 mg/L; Sigma Chemical Co.) for counterselection and cefotaxime (1 mg/L; Sigma Chemical
126 Co.) to select for plasmid-encoded resistance. For detecting plasmids, bacterial cells were
127 embedded in agarose plugs and disrupted by alkaline lysis. Subsequently, the plasmids were
128 converted to the linear forms by incubation with Aspergillus oryzae S1 nuclease (Sigma
129 Chemical Co., St Louis, MO, USA) and were sized by pulsed-field gel electrophoresis (PFGE)
130 as previously described (Ho et al., 2010; Ho et al., 2011b). The replicon types for *E. coli*
131 transconjugants with CTX-M encoding plasmids were determined by a scheme previously
132 described (Carattoli et al., 2005). Eighteen pairs of primers were tested in five multiplex and
133 three simplex PCR assays. The method allowed recognition of the following plasmid
134 incompatibility groups (Inc): FIA, FIB, FIC, FIIA, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K,
135 B/O, X, Y, F. An additional primer pair was used for identification IncFII replicons (Osborn
136 et al., 2000). The FII plasmids were further subtyped by the replicon sequence typing (RST)
137 scheme (Villa et al., 2010). This involved PCR and sequencing of DNA fragments containing
138 the *copA* region of the FII replicon, the iterons-*repE* region of the FIA replicon, the *repB*
139 gene of the FIB replicon and the *copA* region of the FIC replicon. Alleles were assigned to
140 each sequence and the replicon types were expressed according to the FAB formula (Villa et
141 al., 2010). Where a transconjugant could not be obtained, the replicon type for the plasmid
142 encoding CTX-M-14 was determined in the parent strains. In all the isolates, the replicon
143 location in the plasmids was confirmed by hybridization with probes specific for $bla_{\text{CTX-M}}$ and

rep amplified by PCR from different samples. For non-transconjugants, S1-PFGE was used to separate all the plasmids and the plasmid carrying the $bla_{\text{CTX-M}}$ gene was identified by hybridization probe. PCR was used to determine all the plasmid replicons carried by the non-transconjugants. Afterwards, the non-transconjugants were tested by hybridization using probes for all positive replicon types. The *rep* probes that hybridized to the $bla_{\text{CTX-M}}$ carrying plasmid were used to define the replicon type. The four main phylogenetic groups (A, B1, B2, and D) of the *E. coli* isolates were determined by a multiplex PCR assay (Clermont et al., 2000).

PCR-RFLP analysis of IncFII plasmids

The IncFII plasmids were analysed further by a PCR-RFLP scheme previously described (Ho et al., 2011b). The PCR-RFLP scheme involved PCR amplifications of seven loci or regions (designated A, B1, B2, and C to F) with sizes ranging from 1.7 kb to 7.8 kb. Following PCR amplifications, the amplicons were digested with 5 U each of the following restriction enzymes (New England BioLabs) according to the manufacturer's instructions: RsaI (locus A); SmaI (locus B1); SfcI (locus B2); HhaI (locus C); BsmI (locus D); BstEII (locus E); and DrdI (locus F). For each locus, the PCR-RFLP patterns were assigned numbers. Patterns with one or more band differences were assigned different numbers. The profiles for the seven loci were used to designate the pRFLP type. Primers for amplification of the A, B2, C to F loci were those described previously (Ho et al., 2011b). Locus B1 was included in this study to map the region upstream of $bla_{\text{CTX-M-14}}$. The primer pair used to amplify locus B1 was: repA1F (forward), 5'- CGCTCCTTCTGCGCATTGTAA -3' and CTX-M-9F (backward), 5'-CAAAGAGAGTGCAACGGATG -3'(Woodford et al., 2006). The content of the amplified regions in pHK01 were (Ho et al., 2011b): locus A (*finO, orf99-102, repA2, repA3 and repA1*), locus B1 (*repA1, yacABC, yadA, malB, IS903*, $bla_{\text{CTX-M-14}}$), locus B2, ($bla_{\text{CTX-M-14}}$,

IS*ECp1*, *eitD, eitC, eitB*, *eitA, orf18-19,* *parB*), locus C (***parB***, ***parA***), locus D (*orf37*, *orf38-40, ssb, orf42-43, psiB, psiA,* *orf45*), locus E (*traM, J, Y, A, L, E, K, B, P,* *trbD*), and locus F (*traI, X, orf97,* *finO*). The primers were designed within the underlined gene or upstream/downstream of the gene in **boldface.** Plasmid with PCR-RFLP patterns identical to the reference plasmid (pHK01) in three or more loci were considered to be pHK01-like. PCR for the pHK01-associated *malB*, *yac*, *eitA, eitC* and *parA/B* genes was performed using previously described primers (Ho et al., 2011b).

Analysis of *bla*$_{\text{CTX-M-14}}$ genetic environment

The genetic environment may be defined as the sequences flanking the open reading frame of the *bla*$_{\text{CTX-M-14}}$ gene. This includes insertion elements that may be involved in the mobilization and expression of the *bla*$_{\text{CTX-M-14}}$ gene. Based on published studies and sequences deposited in the GenBank database, eight different types of *bla*$_{\text{CTX-M-14}}$ genetic environment have been reported (Table 1). For purpose of description, they were denoted types I to VIII. Monoplex PCRs using different primer pair combinations were used to map the genetic environment to one of the recognized types (Table 1).

Plasmid sequencing

The Illumina Genome Analyzer IIx was used for sequencing of plasmids at approximately 500-fold coverage, as described previously (Ho et al., 2011b). In brief, plasmids were extracted from the transconjugants by using the Qiagen Large Construct kit (Qiagen, Hong Kong). Purified plasmid DNA was fragmented by nebulization. The fragments were amplified and a library was constructed as described previously (Ho et al., 2011c). Based on the qPCR quantified concentration of the barcoded plasmid library, it was diluted to generate approximately 500,000 clusters and seeded with other samples in the same Solexa sample

194 lane. Sequencing run of 76-base pair-end reads was performed according to the
195 manufacturer's recommendations. The Illumina Off-Line Basecaller (version v1.6), WebACT
196 and Geneious Pro (Version 5.0.1, Biomatters Limited, Auckland, New Zealand) softwares
197 were used for bioinformatics analysis (Ho et al., 2011c).
198
199 **Results**
200 In conjugation experiments, the plasmids harbouring CTX-M 14 could be transferred in
201 77.5% (124/160) of the isolates at frequencies of 10^{-6} to 10^{-1} per donor cells. In addition to
202 cefotaxime resistance, the plasmids in 19 transconjugants encoded co-resistance to non-β-
203 lactam antibiotics with profiles involving chloramphenicol, gentamicin, cotrimoxazole and/or
204 tetracycline. These plasmids have origins from 17 faecal (chicken 8, pigs 3, dog 1, humans 5)
205 and two human urine isolates (Supplementary Table S1).
206 Ten different replicons (IncFII, IncFIA, IncFIB, IncI1-Iγ, IncB/O, IncK, IncN, IncA/C,
207 IncHI1, IncHI2), either alone or in combinations were found among the plasmids encoding
208 CTX-M-14 (Table 2). The two most common replicon types were IncFII (38.1%, 61/160) and
209 IncI1-Iγ (15%, 24/160). The frequencies of IncFII replicon were similar among isolates from
210 the three sources: animal faecal (36.2%), human faecal (38.5%) and human urine (40.5%, chi
211 square for trend, $P = 0.7$). Replicon IncI1-Iγ was more common among animal faecal isolates
212 (20.7%) than in human faecal isolates (10.8%) and human urine isolates (13.5%), but the
213 difference was not statistically significant ($P = 0.3$). The replicons of the CTX-M-14
214 encoding plasmids for 19 transconjugants with co-resistance to non-β-lactam antibiotics were
215 as follows: IncFII (n=1), IncFII, FIB (n=2), IncFIB (n=2), IncFII, I1γ (n=2), IncFII, FIA, FIB
216 (n=1), Inc I1γ (n=5), IncB/O (n=2), A/C (n=1), HI2 (n=1) and nontypeable (n=2)
217 Overall, 37.5% (60/160) isolates were phylogenetic group D, 22.5% (36/160) were
218 group B2, 20.6% (33/160) were group A and 19.4% (31/160) were group B1. Analysis of

plasmid replicon distribution showed that the frequency of IncFII replicon in the four phylogenetic groups were similar. The IncFII frequencies for the virulent groups B2 and D were 47.2% (17/36) and 40% (24/60), respectively; that for the commensal groups A and B1 were 39.4% (13/33) and 22.6% (7/31), respectively (virulent vs. commensal groups, $P = 0.1$).

PCR mapping and sequencing of representative products revealed six of the eight reported types of $bla_{\text{CTX-M-14}}$ genetic environment (Table 3 and Supplementary Table S2). One hundred forty-one (88.1%) isolates were found to have a genetic environment consisting of an IS*Ecp1* element upstream and an IS*903* element downstream of the $bla_{\text{CTX-M-14}}$ gene (type II). The proportions of isolates from animal faecal, human faecal and human urine sources with type II CTX-M-14 genetic environment were 89.7%, 87.7% and 86.5%, respectively. In addition to IS*Ecp1* and IS*903*, four other insertion sequences (IS*10*, IS*26*, IS*6100* and IS*CR1*) and class 1 integron were found in the other types of genetic environment (III to V, VII and VIII) in different combinations. Type VII and VIII genetic environments were found to have genes encoding resistance to aminoglycosides (amonoglycoside acetyltransferases, *aacA4* and *aadA2*), chloramphenicol (*cml*), sulphonamides (*sul1*) and/or trimethoprim (dihydrofolate reductase, *dfrA12*).

Table 4 summarized the plasmid subtyping result for the 61 CTX-M-14 encoding IncFII plasmids. RST revealed that the IncFII plasmids had four allelic variants: F2 (n=42), F35 (n=17), F43 (n=1) and F51 (n=1). According to the PCR-RFLP patterns for the seven loci (Figure 1), 32 unique plasmid RFLP (pRFLP) profiles were obtained for the 61 plasmids encoding $bla_{\text{CTX-M-14}}$ (Supplementary Table S3). These included 16 plasmids (group 1) with all seven PCR-RFLP patterns identical to the reference plasmid (pHK01), 36 plasmids (group 2) with identity over three to six loci, and nine plasmids (group 3) without any identical PCR-RFLP pattern or identical patterns in one or two loci only. Hence, 85.2% (52/61) of the

IncFII plasmids (group 1 and 2) were considered to be pHK01-like. These 52 pHK01-like plasmids had their origins from human faecal (*n*=20), human urine (*n*=14) and animal faecal (n=18, including 1 cattle, 1 chicken, 5 pigs, 9 cats, 1 dog and 1 rodent) sources. There were 15 unique pRFLP profiles for plasmids with the F2 and F35 replicon allele each. The presence of the pHK01-associated *malB*, *yac*, *eitA, eitC* and *parA/B* genes was assessed by monoplex PCRs using the purified plasmids as template. The result showed that 90.4% (47/52) of the pHK01-like plasmids were positive for all targets. In contrast, these genes were variably found among group 3 plasmids (Supplementary Table S3). The proportions of animal faecal, human faecal and human urine isolates with pHK01-like plasmids were 31% (18/58), 30.6% (20/65) and 37.8% (14/37), respectively.

The CTX-M-14 encoding plasmids, pHK09 (*E. coli* strain C017e) and pHK17a (*E. coli* strain P0014ST) originating from the faecal samples of a child aged one year in 2007 and a pig in 2008, respectively were sequenced (Ho et al., 2011a; Lo et al., 2010). The sizes of pHK09 (GenBank accession JN087528) and pHK17a (GenBank accession JF779678) were 70382 bp and 70060 bp, respectively. The sequences of the two plasmids were highly similar (>95%) to pHK01 (Hong Kong, 2004, GenBank accession HM355591, pRFLP 1-1-1-1-1-1-1) and the pHK01-like plasmid, pKF3-70 (China, 2006, GenBank accession FJ494913, *in silico* pRFLP 1-1-1-1-1-2-4). Figure 1 showed an alignment of the four plasmids (pHK01, pHK09, pHK17a and pKF3-70) according to the functional regions. Except for some sequence variations and possible insertions/deletions, the four plasmids were virtually identical. With reference to pHK01, sequence variations were found in the *repA4* gene (94.5% similarity, pHK17a), the *ssb* gene (98.9% similarity, pHK17a) and the IS*Ecp1* element (99.8% similarity, pKF3-70). The transfer region from *traM* to *traX* in pHK09 was more similar to pHK01 (99.3%) than to pHK17a (91.6%) or pKF3-70 (91.5%). The regions (*traM* to *traX*) in pHK17a and pKF3-70 were almost identical to each other (99.6% sequence identity).

Sequence variations in the transfer region correlated with the observed difference in the restriction digestion pattern over locus E and F in pHK17a and pKF3-70. In pHK17a, sequence variations within orf37 (encoding an adenine-specific DNA methylase gene) correlated with negative PCR result for locus D. The *yfiA* gene (hypothetical protein), which was found between *traR* and *traC* in pHK01 and pHK09, was absent in pHK17a and pKF3-70. In contrast, an *artA* gene (hypothetical protein), absent in pHK01 and pHK09, was inserted between *traF* and *traQ* in pHK17a and pKF3-70.

Discussion

This study demonstrated the widespread dissemination of pHK01-like plasmids among isolates originating from multiple human and animal sources. The finding showed that 85% (52/61) of the IncFII plasmids and 32.5% (52/160) of all the plasmids encoding CTX-M-14 were pHK01-like. Our previous work showed that pHK01-like plasmids play an important role in the community emergence of CTX-M-14 among urinary *E. coli* in 2004 (Ho et al., 2007; Ho et al., 2011b). The present work extends the observation by showing that pHK01-like plasmids were found among human and animal faecal isolates collected in different time periods. Of note, pHK01-like plasmids appeared to have approximately equal representation among *E. coli* isolates of the four phylogenetic groups. This may partially explain its widespread prevalence in bacteria of both animal and human origin. In Hong Kong, studies have revealed that identical gentamicin resistance genes, integron and cassette arrays were shared by isolates from animal faecal, human faecal and human urinary sources at similar prevalence (Ho et al., 2009; Ho et al., 2010).

Since CTX-M-14 is the only antibiotic resistance gene in most pHK01-like plasmids, persistence and spread of the plasmids cannot be attributed to co-selection by non-β-lactam antibiotics. This single resistance feature is shared by the group of epidemic pCT-like

plasmids (IncK) encoding CTX-M-14 that have been reported in bacteria from human and animals (Cottell et al., 2011). Furthermore, most pHK01-like plasmids had the 1-1 restriction pattern at locus B1 and B2, and were PCR positive for *yac*, *malB*, and *eitA/eitC*. The finding correlated with a conserved array of genes in the variable region (*yac*-*malB*-IS*903*-*bla*$_{\text{CTX-M-14}}$-IS*Ecp1*-*eitABCD*). It is possible that the genes in the variable region may be advantageous to the host bacteria. The toxin-antitoxin plasmid stability system (yac) is recognized to play roles in the stable maintenance of large plasmids while the other transport proteins (malB and eitABCD operon) could provide the host bacteria with some metabolic or virulence advantages (Cheng et al., 2009; Zhao et al., 2009). Interestingly, the yac system and eitABCD operon combination were also found in two major avian pathogenic plasmids, pAPEC-O2-ColV (GenBank accession AY545598) and pAPEC-O1-ColBM (GenBank accession DQ381420) that are widespread among poultry isolates (Johnson et al., 2006).

This study subtyped the IncFII plasmids by several methods. The RST scheme discerned related from unrelated plasmids according to the *copA* sequence, while the pRFLP scheme analysed the plasmid scaffold (Ho et al., 2011b; Villa et al., 2010). Our experience showed that the RST scheme was easy to apply and the discriminatory power was good. However, the RST allele did not consistently predict the plasmid scaffolds. Despite the fact that the two FII alleles (F2 and F35) cluster into two distinct replicon subgroups (Villa et al., 2010), plasmids of the two replicon alleles could share highly similar pRFLP profiles. Complete sequencing of two other pHK01-like plasmids confirmed that variations in the pRFLP profiles correlate with DNA insertion, deletion, point mutations and possible homologous recombination. The finding suggests that multiple genetic processes are involved in the evolution of the pHK01-like plasmids.

Among isolates of all sources, the predominant genetic environment of *bla*$_{\text{CTX-M-14}}$ consisted of an upstream IS*Ecp1* and a downstream IS*903* (type II). This type of genetic

environment was similarly prevalent among the IncFII and non-IncFII plasmids. This study also revealed the presence of several other mobile genetic elements (IS*10*, IS*26*, IS*6100*, IS*CR1* and class 1 integron) in association with $bla_{\text{CTX-M-14}}$ and/or IS*Ecp1*/IS*903* in six different combinations. Thus, many genetic elements might be involved in the mobilization of $bla_{\text{CTX-M-14}}$ into different plasmids (Barlow et al., 2008). In the present study, almost all recognized genetic environment types were found. This could possibly be due to the diverse origins of animals.

This study has limitations. Since pigs and chicken from different farms had been mixed at the time of sampling, the geographic origins of these samples could not be traced individually. Therefore, the observed variations in CTX-M-14 genetic environment and divergence in plasmid sequences could be partly related to mixed origins of the samples from local farms and importation from mainland China. Secondly, only two plasmids were completely sequenced. As a result, the full spectrum of sequence divergence in the plasmids could not be comprehensively resolved. Nonetheless, multiple pRFLP profiles involving variations in different loci were found for isolates that originated from the same animal and human sources. The finding highlights plasticity among related plasmids in different modules of the plasmid backbones and the $bla_{\text{CTX-M-14}}$ genetic environment. In the future, additional plasmids with farm and geographical origins clearly defined should be investigated to clarify the epidemiologic pattern of different plasmid sequences. Preferably, a larger number of pHK01-like plasmids from different time periods should be completely sequenced to delineate how plasmid sequence divergence correlates with the epidemiologic information and how that changes over time.

Conclusion

This study showed that the pHK01-like plasmids encoding CTX-M-14 were shared among *E. coli* isolates collected in different time periods from multiple human and animal sources. Public health authorities should adopt measures to reduce the direct and indirect transfer of resistant bacteria or resistance determinants within animal populations, from animals to human and vice versa (Codex Alimentarius Commission, 2005).

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Transparency Declaration

Conflicts of interest: Nothing to declare.

Appendix A

Supplementary data

Table 1

Oligonucleotide primers for PCR mapping of CTX-M-14 genetic environment

Primer name[a, b]	Direction	Nucleotide sequence (5' to 3')	Position	Accession No.	Amplicon size (bp)	Source
IS*Ecp1*U2	F	AATACTACCTTGCTTTCTGA	7650-7669	HM355591	1464	(Saladin et al., 2002)
M9L	B	CCCTTCGGCGATGATTCTC	6206-6224			(Saladin et al., 2002)
IS*Ecp1*U1	F	AAAAATGATTGAAAGGTGGT	7210-7229	HM355591	366	(Saladin et al., 2002)
CTX-M-9B	B	ATTGGAAAGCGTTCATCACC	6864-6883			(Woodford et al., 2006)
IS*CR1*F	F	GCCACCAACCCGACCAGAC	4001-4019	EF450247	2019	This study
CTX-M-9B	B	ATTGGAAAGCGTTCATCACC	6000-6019			(Woodford et al., 2006)
tnpIS*26*-R	F	AACTCTGCTTACCAGGCG	1413-1430	GQ385314	1507	This study
CTX-M-9B	B	ATTGGAAAGCGTTCATCACC	2900-2919			(Woodford et al., 2006)
ISEcp_uw	F	AACATCAAACGAATCGACCG	799-818	EU136400	1468	This study
M9L_dw	B	CACCTGCGTATTATCTGCGG	2247-2266			This study
Int-F	F	GCCACTGCGCCGTTACCACC	322-341	EF450247		(Ho et al., 2010)
Int1-285B	B	GCACAGCACCTTGCCGTAGAA	66-86		276	This study
CTX-M-9B	B	ATTGGAAAGCGTTCATCACC	6000-6019		5698	(Woodford et al., 2006)

M9U	F	ATGGTGACAAAGAGAGTGCA	7056-7075	HM355591	1265	(Saladin et al., 2002)
IS*903*D-5811R	B	TAACCGACTTTGCCCGCCTG	5811-5830			This study

[a] Based on published studies and sequences deposited in the GenBank database, eight different types of *bla*$_{CTX-M-14}$ genetic environment have been reported. Type I (IS*Ecp1* - *bla*$_{CTX-M-14}$) consisted of an upstream IS*Ecp1* but the downstream IS*903* was not detected by PCR (Kim et al., 2011). However, the presence of a truncated IS*903* has not been confirmed by plasmid sequencing. The GenBank accession numbers for the other types were HM355591 (type II, IS*Ecp1* - *bla*$_{CTX-M-14}$ - IS*903*), EU136400 (type III, ΔIS*Ecp1* - IS*10* - *bla*$_{CTX-M-14}$ - IS*903*), GQ385314 (type IV, IS*26* - ΔIS*Ecp1* - *bla*$_{CTX-M-14}$ - IS*903*), AB180674 (type V, IS*26* - ΔIS*10* - *bla*$_{CTX-M-14}$ - IS*903*), EU056266 (type VI, Class 1 integron- *orf5* - IS*6100* -IS*CR1* - ΔIS*Ecp1* - *bla*$_{CTX-M-14}$ - IS*903*), FQ482074 (type VII, Class 1 integron- o*rf1* -IS*Ecp1* - *bla*$_{CTX-M-14}$ - IS*903*) and EF450247 (type VIII, Class 1 integron- IS*CR1*-*bla*$_{CTX-M-14}$ - IS*903*). The class 1 integron include type VI and VIII contain *intl1-dfrA12-orfF-aadA2-qacEΔ1-sul1* (type VI and VIII) or *Δintl1-aacA4-cml-qacEΔ1-sul1* (type VII).

[b] Monoplex PCRs using different primer pair combination was used to map the genetic environment to one of the eight recognized types. All the assigned types yielded amplicons of the expected sizes. Representative amplicons were sequenced for confirmation.

Table 2

Replicon types for plasmids encoding CTX-M-14 analysed in this study

Replicon type[a]	No. of isolates	Plasmid sizes, kb	No. (%) according to source		
			Animal faecal	Human faecal	Human UTI
FII	61	55-100	21 (36.2)	25 (38.5)	15 (40.5)
FII, FIB	7	100-150	3	3	1
FIB	6	55-190	1	3	2
FII, I1-Iγ	3	70-100	1	2	-
FIA	3	55-100	-	1	2
FII, FIA	2	100-130	-	2	-
FIA, FIB	1	80	1	-	-
FII, FIA, FIB	1	150	-	1	-
I1-Iγ	24	40-120	12 (20.7)	7 (10.8)	5 (13.5)
B/O	10	60-140	5	5	-
K	6	80-100	-	5	1
Others[b]	36	50-250	14	11	11
Total	160	-	58	65	37

[a] The replicon types were determined by probe hybridization in the transconjugants (n=84) or the parent strains (n=76).

[b]These included N (n=3), A/C (n=1), HI1 (n=1), HI2 (n=1) and nontypeable (n=30).

Table 3

CTX-M-14 genetic environment of *E. coli* isolates from humans (*n*=102) and animals (*n*=58), Hong Kong.

Type[a]	Genetic environment of $bla_{\text{CTX-M-14}}$	No (%)		
		Animal-faecal	Human-faecal	Human-urine
II	IS*Ecp1* - $bla_{\text{CTX-M-14}}$ - IS*903*	52 (89.7)	57 (87.7)	32 (86.5)
III	ΔIS*Ecp1* - IS*10* - $bla_{\text{CTX-M-14}}$ - IS*903*[b]	-	4	1
IV	IS*26* - ΔIS*Ecp1* - $bla_{\text{CTX-M-14}}$ - IS*903*	3	1	-
V	IS*26* - ΔIS*10* - ΔIS*Ecp1*- $bla_{\text{CTX-M-14}}$ - IS*903*[c]	1	-	1
VII	Class 1 integron- o*rf1* -IS*Ecp1* - $bla_{\text{CTX-M-14}}$ - IS*903*	2	1	3
VIII	Class 1 integron- IS*CR1*-$bla_{\text{CTX-M-14}}$ - IS*903*	-	2	-
Subtotal		58	65	37

[a] The Genbank accession numbers were as follows: HM355591 (type II), EU136400 (type III), GQ385314 (type IV), AB180674 (type V), EU056266 (type VI, Class 1 integron- *orf5* - IS*6100* -IS*CR1* - ΔIS*Ecp1* - $bla_{\text{CTX-M-14}}$ - IS*903*), FQ482074 (type VII) and EF450247 (type VIII). The class 1 integron includes type VI and VIII containing *intl1-dfrA12-orfF-aadA2-qacEΔ1-sul1* (type VI and VIII) or *Δintl1-aacA4-cml-qacEΔ1-sul1* (type VII).

[b] The ΔIS*Ecp1* was interrupted by an IS*10* element.

[c] The ΔIS*Ecp1* element was very short and limited to a putative IS*Ecp1* promoter.

Table 4

Characteristics of the IncFII plasmids encoding *bla*$_{CTX-M-14}$ in 61 *Escherichia coli* isolates from humans and animals.

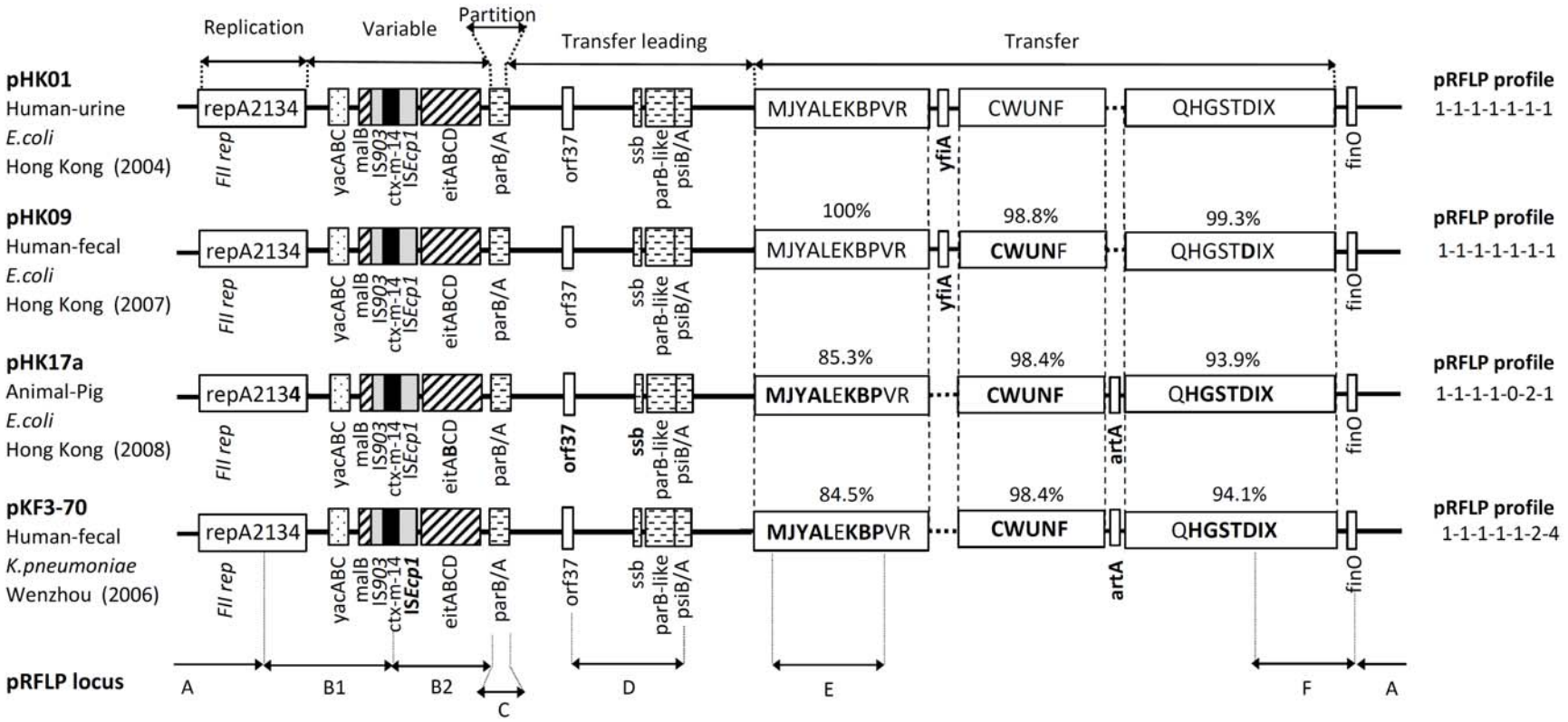
	No of plasmids[a]			
	Group 1	Group 2	Group 3	All
Number of plasmids	16	36	9	61
FAB formula				
F2:A-:B-	16	26	-	42
F35:A-:B-	-	10	7	17
F43:A-:B-	-	-	1	1
F51:A-:B-	-	-	1	1
PCR profile				
malB-yac-eitA-eitC-parAB	16	31	2	49
Others[b]	-	1	5	6
None	-	4	2	6
Source				
Human UTI	5	9	1	15
Human faecal	4	16	5	25
Chicken	1	-	-	1
Cattle	1	-	-	1
Pig	1	4	1	6
Cats	3	6	1	10
Dogs	0	1	-	1
Rodents	1	-	1	2

[a]The plasmids were categorised according to their similarity to the reference plasmid, pHK01: group 1, all seven PCR-RFLP patterns were identical to reference; group 2, three to six restriction patterns were identical; and group 3, less than three restriction patterns were identical.

[b]Including the following profiles: *yac-eitA-eitC-parAB* (*n*=2), *yac-eitC-parAB* (*n*=1), *mal-eitA-eitC* (*n*=1), *parAB* (*n*=2).

FIGURE 1. **Schematic representation of pHK09 and pHK17a and comparison with two other CTX-M-14 encoding plasmids, pHK01 and pKF3-70.**

The sequence of pHK01 was used as the reference for all comparisons. The five functional regions were indicated by arrows on top while the seven sets of long-range PCR targets (locus A to locus F) were indicated by arrows in the bottom. The genes and ORF of interest were indicated in boxes and annotated. Potential deletions were indicated by dotted line. The *rep* genes in the replication and *tra* genes in the transfer region were indicated by white boxes with number and capital letters, respectively. The percentage identity over each indicated block in the transfer region were given above the boxes. The genes or ORFs with sequence variations were indicated in **boldface**. *Orf37* is named according to pHK01. Its counterpart is *orf35* in pHK09 and pHK17 and *orf81* in pKF3-70. The boxes according to shading patterns were: dotted lines (partition-associated genes, *parA/B, ssb, parB-like, psiA/B*), dots (toxin-antitoxin genes, *yacABC*), oblique lines (transportation-related proteins, *malB* and *eitABCD*), gray (insertion sequences, IS*903* and IS*Ecp1*) and black (resistance gene, $bla_{\text{CTX-M-14}}$). The plasmids' origins and their pRFLP profiles were labelled on the two sides..


Replication
Variable
Partition
Transfer leading
Transfer
pHK01
Human-urine
E.coli
Hong Kong (2004)
pHK09
Human-fecal
E.coli
Hong Kong (2007)
pHK17a
Animal-Pig
E.coli
Hong Kong (2008)
pKF3-70
Human-fecal
K.pneumoniae
Wenzhou (2006)
repA2134
FII rep
yacABC
malB
IS903
ctx-m-14
ISEcp1
eitABCD
parB/A
orf37
ssb
parB-like
psiB/A
MJYALEKBPVR
CWUNF
QHGSTDIX
yfiA
artA
finO
100%
98.8%
99.3%
85.3%
98.4%
93.9%
84.5%
98.4%
94.1%
pRFLP profile
1-1-1-1-1-1-1
1-1-1-1-1-1-1
1-1-1-1-0-2-1
1-1-1-1-1-2-4
pRFLP locus
A
B1
B2
C
D
E
F
A

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Ref Type: Generic

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