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Prevalence and characterization of hybrid *bla*$_{\text{CTX-M}}$ among *Escherichia coli* isolates from livestock and other animals

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Highlights

- We investigated the prevalence of hybrid *bla*$_{\text{CTX-M}}$ among isolates from different hosts.
- Hybrid *bla*$_{\text{CTX-M}}$ of subgroup 1 and 9 genes were found in 14 (5.6%) isolates.
- There were variations in prevalence by host sources.
- Hybrid *bla*$_{\text{CTX-M}}$ genes were mainly harbored on IncI1 or IncI2 plasmids.
- Our plasmids were highly similar to plasmids previously reported from China.

Abstract

This study investigated 248 extended-spectrum β-lactamase-producing *Escherichia coli* isolates from 2012-2013 for hybrid bla_{CTX-M} genes. bla_{CTX-M} genes were detected in 228 isolates of which 14 isolates were hybrid bla_{CTX-M}-positive (six $bla_{CTX-M-123}$, six $bla_{CTX-M-64}$ and two $bla_{CTX-M-132}$). The 14 hybrid bla_{CTX-M}-carrying isolates (eight from chickens, two each from pigs and cattle, one each from dog and rodent) were genetically diverse. All but two hybrid bla_{CTX-M} were carried on IncI1 (five $bla_{CTX-M-123}$) and IncI2 (six $bla_{CTX-M-64}$ and one $bla_{CTX-M-132}$) plasmids. Our IncI1 and IncI2 plasmids had pHNAH4-1-like and pHN1122-1-like RFLP patterns, respectively. Genetic relatedness of the plasmids to pHNAH4-1 and pHN1122-1 were confirmed by complete sequencing of three plasmids, pCTXM123_C0996, pCTXM64_C0967 and pCTXM132_P0421. Plasmids closely related to pHNAH4-1 and pHN1122-1, and carrying different bla_{CTX-M} alleles have been reported from multiple geographic areas in China previously. The findings highlighted the wide dissemination of hybrid bla_{CTX-M} variants in different parts of China.

1. Introduction

Gram-negative bacteria producing CTX-M type extended-spectrum β-lactamases (ESBLs) are a major public health threat worldwide and have been found to be highly prevalent in human and animals (Woerther et al., 2013; Ho et al., 2012a). CTX-M sequence variants can be divided into six clusters (CTX-M-1, -2, -8, -9, -25 and KLUC subgroups) with less than 90% identity between cluster and more than 95% identity within clusters (D'Andrea *et al.*, 2013). Point mutation is the predominant mechanism from which new CTX-M alleles arise. However, it is recently recognized that CTX-M variants could also evolve by homologous recombination of two different CTX-M genes (He et al., 2013; Nagano et al., 2009).

In 2009, a novel variant, CTX-M-64 was found in *Shigella sonnei* from a patient with diarrhea after a trip to China (Nagano *et al.*, 2009). Sequence analysis of CTX-M-64 revealed that it is chimeric with CTX-M-15-like regions in the N- and C-terminal moieties and CTX-M-14-like region in the central portion (Nagano *et al.*, 2009). Currently, four hybrids of CTX-M-14-like and CTX-M-15-like subgroup enzyme have been reported. CTX-M-64, CTX-M-123 and CTX-M-132 are hybrid enzymes with CTX-M-15-like regions in the N- and C-terminal moieties and CTX-M-14-like region in the central portion and have been identified in *E. coli* isolates originating from pets, food animals and retailed meat in China (He et al., 2013; Li et al., 2010; Sun et al., 2010). CTX-M-137, a hybrid with

CTX-M-14-like N terminus and CTX-M-15-like C terminus, was identified from southern China in an *E. coli* clinical isolate in a patient with urinary tract infection (Tian *et al.*, 2014).

Since these CTX-M variants are chimeric, widely used subgroup-specific PCR assays for detection of CTX-M genes may failed to identify their presence (Woodford et al., 2006; Saladin et al., 2002). Therefore, information on the prevalence and distribution of the CTX-M hybrids are limited. Hence, this study was conducted to investigate the prevalence and molecular epidemiology of the CTX-M hybrid enzymes in *E. coli* from human and animal sources. In China, plasmid-mediated fosfomycin resistance has been reported to be emerging among CTX-M-producing isolates from animals (Hou et al., 2012; Ho et al., 2013b). Therefore, we also investigated the plasmid-mediated fosfomycin resistance determinants among the isolates producing CTX-M hybrids

2. Materials and methods.

2.1 Bacterial strains and susceptibility testing

A total of 340 nonduplicated, consecutive urinary *E. coli* isolates were collected from four clinical microbiology laboratories over a two week period, from May to June 2013 (Ho et al., 2015). The laboratories together served about a quarter of the Hong Kong populations in different geographic districts. The inclusion criteria were: (1) patient age 18 years or above, (2) mid-stream urine specimen, and (3) significant growth of $\geq 10^5$ CFU/ml. Among the 340

urinary isolates, 78 isolates were ESBL-producing. All ESBL-producing isolates were included in this study.

Animal isolates were obtained from an ongoning antimicorbial surveillance program during September 2012-August 2013 (Ho et al., 2011a; Ho et al., 2013b). For cattle and pigs, rectal swabs were obtained from fresh carcasses in a centralized slaughterhouse in Hong Kong. Samples from chicken were obtained while the animals were temporarily held for inspection before sale in wet markets in Hong Kong. All the cattle were imported from mainland China. Pigs and chickens included animals reared in local farms and those imported from mainland China. Samples from stray dogs, stray cats and rodents were collected by trained staff at the Governmental Animal Management Centres. The stray animals and wild rodents were captured from urban areas from all over Hong Kong. The animals were sampled in batches and each batch was obtained on a different date: chickens (20 animals per batch), cattle (10 animals per batch), pigs (2–7 animals per batch), stray cats (1–10 animals per batch), stray dogs (1–10 animals per batch) and urban rodents (2–23 animals per batch). Samples (approximately 0.1 g of feces) were seeded with Dacron swabs onto MacConkey agars supplemented with 2 μg/ml cefotaxime or 2 μg/ml ceftazidime, as previously described (Ho *et al.*, 2011a). In brief, 534 ESBL-producing *E. coli* isolates were recovered from 1090 animals including 241 cats (52 batches), 200 chickens (10 batches), 255 dogs (72 batches), 100 cattle (10 batches), 112 pigs (32 batches), and 182 rodents (11 batches). A subset of 170

ESBL-producing isolates (40 from dogs, 14 from cats, six rodents, 38 from chickens, 56 from pigs and 16 from cattle) were chosen randomly according to sampling date and animal type for testing. The six rodent isolates include five from *Rattus norvegicus* and one from *R. rattus*.

In total, 248 ESBL-producing isolates including 78 isolates from patients and 170 isolates from animals were investigated. The isolates were stored in Microbank (Pro-Lan Diagnostics) at -80 ^{o}C until testing. A disc diffusion method was used for susceptibility testing and the results were interpreted according to the Clinical and Laboratory Standrads Institute guidelines (CLSI, 2014). A double-disc synergy test was used for detection of ESBLs (Ho et al., 1998). This study was approved by the University of Hong Kong's Committee on Animal Ethics (CULATR).

2.2 Genotyping

PCR assays were used to assign the *E. coli* isolates to phylogenetic groups A, B1, B2, C, D, E and F (Clermont et al., 2013). Multilocus sequence typing (MLST) was carried out and results were analysed using the University of Warwick scheme (http://mlst.warwick.ac.uk/mlst/).

2.3 Detection of antibiotic resistance genes

PCR and sequencing were used to investigate the antibiotic resistance genes. Primers for the CTX-M subgroups (consensus, *bla*$_{CTX-M1G}$, *bla*$_{CTX-M2G}$, *bla*$_{CTX-M8G}$, *bla*$_{CTX-M9G}$, and *bla*$_{CTX-M25G}$) have been described previously (Ho *et al.*, 2011b; Ho *et al.*, 2012a). Two primer pairs were designed for the detection of hybrid *bla*$_{CTX-M}$ by monoplex PCR assays: CTXM-1F and CTXM9-506B for *bla*$_{CTX-M-64/123/132}$-like hybrid genes, and CTXM9-F and CTXM1-600B for *bla*$_{CTX-M-137}$-like hybrid genes (Supplementary file, Table S1). The CTX-M allele was determined by sequencing the full length of the *bla*$_{CTX-M}$ gene using different combination of primers (Ho *et al.*, 2012a; Ho *et al.*, 2012b). Previously described primers were used to investigate for the presence of the plasmid-mediated fosfomycin resistance genes (*fosA*, *fosA3*, *fosA4* and *fosC2*) (Ho et al., 2013a).

2.4 Plasmid studies.

Transferability of hybrid *bla*$_{CTX-M}$ in all the PCR-positive isolates was investigated by filter mating using *E coli* J53 (azide-resistant) as the recipient (1:2 ratio). Transconjugants were selected on MacConkey agar containing 150 μg/ml sodium azide, 1 μg/ml cefotaxime (Sigma) (Ho *et al.*, 2012b). The transfer frequencies were expressed as the number of transconjugants per donor cell (T/D) as previously described (Lo *et al.*, 2014). Plasmids were sized by the S1 nuclease-PFGE method (Ho *et al.*, 2005). The replicon types for the *E. coli* transconjugants with hybrid *bla*$_{CTX-M}$-carrying plasmids were determined by an expanded

PCR-based replicon typing scheme which recognizes HI1, HI2, I1, I2, L/M, N, N2, FIA, FIB, FIC, FIIA, F, K, B/O, W, Y, P, A/C, A/C2, T, X1, X2, X3 and X4 replicons (Carattoli et al., 2005; Lo et al., 2014). Identification of the plasmid replicons was confirmed by sequencing of the PCR products. In all isolates, plasmid location of the $bla_{\text{CTX-M}}$ and replicons were confirmed by hybridization using specific PCR products as probes (Ho *et al.*, 2011a). The IncI1 and IncI2 plasmids carrying hybrid $bla_{\text{CTX-M}}$ in the transconjugants or transformants were further analyzed by restriction fragment length polymorphism (RFLP) (Ho *et al.*, 2011b). Purified plasmids were digested separately with *ApaL*1 and *EcoR*I (Takara, Dalian, China) in accordance with the manufacturer's recommendation. Plasmid MLST (pMSLT) typing (http://pubmlst.org/plasmid/) was used to further characterized the IncI1 plasmids.

2.5 Plasmid sequencing.

One of each of the plasmids encoding $bla_{\text{CTX-M-64}}$, $bla_{\text{CTX-M-123}}$, *and* $bla_{\text{CTX-M-132}}$ were chosen for complete plasmid sequencing. pCTXM64_C0967 and pCTXM132_P0421 were both IncI2 while pCTXM123_C0996 was IncI1 by PCR-based replicon typing. The three plasmids were chosen from the predominant plasmid incompatibility groups (IncI1 or IncI2) and RFLP profiles. Plasmid DNA was prepared as described previously from *E. coli* J53 transconjugant carrying each as the only plasmid (Ho et al., 2011c). All of them were sequenced by Ion Torrent system (Life Technologies) at approximately 500 fold coverage

according to the manufacturer's recommendation. The reads were assembled by the Velvet assembler (version 1.2.10). Gaps in the plasmids were closed by additional PCRs and Sanger sequencing. Fully assembled plasmid sequences were verified by RFLPs of the plasmids with *ApaL*1 and *Ecor*I. The RFLP patterns were then compared with the *in silico* restriction analysis result. The plasmids were annotated using the RAST Server and each predicted open reading frame (ORF) was further blasted against the National Center for Biotechnology Information (NCBI) non-redundant protein database using BLASTX. The prototype plasmids for IncI1 (R64, GenBank accession AP005147) and IncI2 (R721, AP002527) were used as references in annotation. WebACT and Geneious Pro (version R7; Biomatter Limited, Auckland, New Zealand) softwares were used for bioinformatics analysis). The nucleotide sequences have been deposited in the GenBank: pCTXM64_C0967 (KP091735), pCTXM123_C0996 (KP198616), and pCTXM132_P0421 (KP198615).

3. Results

3.1 Prevalence of bla_{CTX-M} subgroups and bla_{CTX-M} hybrid

PCR showed that 91.9% (228/248) of the ESBL-producing isolates were $bla_{\text{CTX-M}}$ positive (Table 1), among which 26.3%-50% and 16.7%-67.9% had $bla_{\text{CTX-M9G}}$ and $bla_{\text{CTX-M1G}}$, respectively, depending on the host source. Ten (4.0%) isolates were PCR positive for both $bla_{\text{CTX-M9G}}$ and $bla_{\text{CTX-M1G}}$, and 14 isolates (5.6%) were PCR positive for

bla$_{\text{CTX-M-64/123/132}}$-like hybrid genes. All isolates were negative for *bla*$_{\text{CTX-M-137}}$-like hybrid genes. None carried *bla*$_{\text{CTX-M2G,}}$ *bla*$_{\text{CTX-M8G}}$ and *bla*$_{\text{CTX-M25G}}$. The prevalence of hybrid *bla*$_{\text{CTX-M}}$ was 8.2% among all animal isolates (Table 1), highest among isolates from chickens (21.1%), following by rodents (16.7%), cattle (12.5%), pigs (3.6%) and dogs (2.5%). No hybrid *bla*$_{\text{CTX-M}}$ was identified among isolates from cats and human.

3.2 Characteristics of hybrid *bla*$_{\text{CTX-M}}$ carrying *E. coli* isolates and plasmids

The 14 isolates carrying hybrid *bla*$_{\text{CTX-M}}$ were further characterized. Phylogenetic group analysis revealed that five isolates belonged to group A, four isolates to group E, two to group F, two to B1 and one to group D (Table 2). MLST revealed that the clones of the hybrid *bla*$_{\text{CTX-M}}$ carrying *E. coli* isolates were diverse. Three isolates were ST1011 while the remaining ones were singletons. The alleles of the hybrid *bla*$_{\text{CTX-M}}$ included *bla*$_{\text{CTX-M-64}}$ (six isolates), *bla*$_{\text{CTX-M-123}}$ (six isolates) and *bla*$_{\text{CTX-M-132}}$ (two isolates).

In conjugation experiments, the plasmids harbouring hybrid *bla*$_{\text{CTX-M}}$ could be transferred in 11 of the 14 isolates at frequencies of 10^{-2} to 10^{-5} per donor cells to the recipient J53 strain. No transconjugants were obtained for the remaining three isolates despite repeated attempts. For the three isolates with no transconjugants, one (strain C0925A) had transfer of the plasmid to DH5alpha recipient by transformation. S1-PFGE showed that only a single plasmid was transferred in all the transconjugants (n=11) and transformant (n=1) (Table 2).

Five transconjugants carrying *bla*$_{\text{CTX-M-123}}$ had co-transfer of resistance to fosfomycin. Plasmids carrying hybrid *bla*$_{\text{CTX-M}}$ were approximately 70-110 kb in size. The replicon types of the hybrid *bla*$_{\text{CTX-M}}$ plasmids were IncI2 (n=7), IncI1 (n=5) and nontypable (n=2). The plasmid locations of the replicon and *bla*$_{\text{CTX-M}}$ genes were confirmed by hybridization. All six IncI2 plasmids carrying *bla*$_{\text{CTX-M-64}}$ were subjected to *ApaL*1 or *Ecor*1 digestion and identical or similar patterns were obtained. All five IncI1 plasmids carrying *bla*$_{\text{CTX-M-123}}$ were of plasmid ST108 (allelic profile 2/13/5/1/2) and they had identical or similar patterns after *ApaL*1 or *Ecor*1 digestion (Table 2).

3.3 Analysis of plasmid sequences

Three plasmids from the transconjugants were completely sequenced, including pCTXM123_C0996 (IncI1, 108,435 bp, GC 50.7%, 130 putative ORFs), pCTXM64_C0967 (IncI2, 62,194 bp, GC 42.1%, 86 putative ORFs), and pCTXM132_P0421 (IncI2, 63,124 bp, GC 42.1%, 89 putative ORFs). The plasmid backbone of pCTXM123_C0996 shares high sequence homology with plasmids within the IncI1 group (Figure 1), such as the reference R64 with genes encoding replication (*repY* and *repZ*), maintenance and stability (*yefA*, *parA/B*, *impB/A/C*, *yfhA*, *pndA/C*), conjugation (24 *tra* and 3 *trb* genes), shufflon region (*rci* gene) and type IV pilus formation (13 genes, *pilI* to *pilU*). Overall, pCTXM123_C0996 share 96.9% coverage and 93.1% identity with another IncI1, *bla*$_{\text{CTX-M-123}}$-carrying plasmid,

pHNAH4-1 (KJ125070, 108,658 bp) obtained from a chicken *E. coli* isolate in Anhui, China. The resistance genes in pCTXM123_C0996 were found within two variable regions with length of 9.2 kb and 5.8 kb. The 9.2 kb region was flanked by an ΔIS*6100* and an ΔIS*26* at the two ends, and includes genes encoding macrolide (*mph2*) and fosfomycin (*fosA3*) resistance. The 5.8 kb variable region (Figure 2), including the hybrid $bla_{\text{CTX-M-123}}$ and a 2721 bp sequence matching IncI2 backbone was flanked by direct repeat sequences of 6 bp (TTATAC). The spacer length between the right inverted repeat (IRR) of the IS*Ecp1* and $bla_{\text{CTX-M-123}}$ is 45 bp. The *orf477* downstream was followed by a 112-bp fragment of a DNA modification methylase from IncA/C backbone. The sequence and context of this 5811 bp putative transposition unit, except for carrying different $bla_{\text{CTX-M}}$ alleles completely matched a region in eight of the IncI2 plasmids showed in Figure 1. The finding suggested that IS*Ecp1* may have mobilized a fragment of IncI2 backbone in addition to $bla_{\text{CTX-M-123}}$ and inserted it in an IncI1 backbone at the AT-rich region between the ybeB and yedA genes (Figure 2). The length of the intergenic sequences between *ybeB* and *yedA*, interrupted by the 5811 bp IS*Ecp1*-transposition unit was 471 bp which was 100% identical to the uninterrupted intergenic sequence in the Inc1I plasmid pIFM3801 (KF787110).

The two IncI2 plasmids (pCTXM64_C0967 and pCTXM132_P0421) shared a high degree of homology to each other and to other completely sequenced IncI2 plasmids, such as the reference R721 (Figure 1). Blast search identified another six IncI2 plasmids carrying

bla$_{\text{CTX-M-15/55/64/132}}$ that have almost identical arrays of ORFs and genes to our two IncI2 plasmids. The sequence of pCTXM64_C0967 and pCTXM132_P0421 have the highest identities to pHNAH46-1 (KJ020576, 100% coverage, 96.7% identity) and pHNLDH19 (KM207012, 100% coverage, 98.1% identity), respectively (Figure 1). The two IncI2 plasmids contained the following functional regions, which were common to all IncI2 plasmids: replication (*repR* and *repA*), maintenance and stability (*yaeC, yafA, parA, topB*), conjugation (*nikABC* genes, *tra* genes), shufflon region (*rci* gene) and type IV pilus formation (with 11 genes, *pilV* to *pilL*). In our two IncI2 plasmids, as were the other six plasmids, the hybrid *bla*$_{\text{CTX-M}}$ was the only resistance gene present. In the eight *bla*$_{\text{CTX-M}}$-carrying IncI2 plasmids, the 3090 bp ΔIS*Ecp1*-*bla*$_{\text{CTX-M}}$-Δorf477-IncA/C transposition unit, flanked by a 5 bp direct repeat (GAAAA) was inserted at an identical position between the *yajA* gene and *orf16* (a hypothetical protein) (Figure 2). In this region, the immediate *bla*$_{\text{CTX-M}}$ genetic context was 100% identical in the eight plasmids but different *bla*$_{\text{CTX-M}}$ alleles were carried: *bla*$_{\text{CTX-M-15}}$ (pHNY2), *bla*$_{\text{CTX-M-55}}$ (pHN1122-1, p1081-CTX-M and pSTH21), *bla*$_{\text{CTX-M-64}}$ (pHNAH46-1, pCTX-M64_C0967) or *bla*$_{\text{CTX-M-132}}$ (pHNLDH19, pCTX-M132_P0421) (Figure 1 and 2). In all eight *bla*$_{\text{CTX-M}}$-carrying IncI2 plasmids, the length of the intergenic sequences between *yajA* gene and *orf16*, interrupted by a IS*Ecp1*-transposition unit is 86 bp which is 100% identical to the uninterrupted intergenic sequence in other Inc2 plasmids not carrying *bla*$_{\text{CTX-M}}$, such as pBK15692 (KC845573)

(Figure 2).

4. Discussion

This study demonstrated the widespread occurrence of hybrid CTX-Ms among isolates from diverse host sources but with wide variations in terms of prevalence. Relatively high frequencies (12.5%-21.1%) were found among ESBL-producing isolates from chickens, rodents and cattle while the frequencies in pigs and dogs were low (2.5%-3.6%). No hybrid CTX-M was identified in isolates from cats and humans. Three different CTX-M variants which are hybrids of CTX-M-1 subgroup and CTX-M-9 subgroup were detected. It is notable that CTX-M-64, CTX-M-123 and CTX-M-132 described so far have been linked to sources in different parts of China. CTX-M-64 has previously been found in a patient after a trip to China and in several animal species (chicken, cats and dogs) from Sichuan and Guangdong provinces, China (Li et al., 2010; Nagano et al., 2009; Sun et al., 2010). CTX-M-123 has been detected in one isolate each from chicken and pig in Anhui and Guangdong provinces, respectively while CTX-M-132 has been detected in meat products in Beijing (He et al., 2013). This study extended those observations and revealed their presence in cattle and wild rodents (*R. norvegicus*).

Our findings showed that the great majority of hybrid $bla_{\text{CTX-M}}$ genes were carried on IncI1 and IncI2 plasmids. The possibility of horizontal plasmid transfer is supported by the

detection of highly similar *bla*$_{CTX\text{-}M\text{-}123}$-carrying IncI1 plasmids and *bla*$_{CTX\text{-}M\text{-}64}$-carrying IncI2 plasmids in different *E. coli* strains. The finding that three of the six IncI2 plasmids carrying *bla*$_{CTX\text{-}M\text{-}64}$ genes are from the same *E. coli* ST type (ST1011) and all from chickens (Table 2) raises the possibility there was clonal spread of the strains. The presence of the same *E. coli* ST type or the highly similar plasmids carrying hybrid *bla*$_{CTX\text{-}M}$ over different time periods suggested that the resistance determinants were already endemic in the animals. IncI1 plasmids are common (10-20%) among *E.. coli* isolates from humans and animals (Lo et al., 2014) and are noted for their possession of many resistance genes, especially those encoding the CTX-M type ESBLs (Carattoli, 2013). Here, we further showed that this plasmid type has captured the emerging fosfomycin resistance *fosA3* gene, which often co-harbor with *bla*$_{CTX\text{-}M\text{-}3/14/55/65}$ on IncFII plasmids (Ho et al., 2013b; Ho et al., 2013a). The success of IncI1 plasmid has been attributed to its very efficient conjugation system (*tra*, *trb* and *pil* loci) (Carattoli, 2013). In addition, the type IV pilus encoded by the *pil* locus contributes to the virulence of Shiga-toxigenic *E. coli* by promoting epithelial cell adherence and adherence to abiotic surfaces (Garcia-Fernandez et al., 2008; Dudley et al., 2006). IncI2 plasmids have scaffold similar to IncI1 plasmids, including the *tra* and *pil* systems. Owning to the inability of the PBRT to identify IncI2 (Carattoli et al., 2005), information on the prevalence of this plasmid type is more limited. Our whole plasmids alignment revealed that IncI2 plasmids carrying hybrid *bla*$_{CTX\text{-}M}$ were highly similar to plasmids carrying *bla*$_{CTX\text{-}M\text{-}15}$ in pets and food

animals (pHN1122-1 like), other *bla*$_{CTX-M-55}$-carrying plasmids detected in patient with *Shigella sonnei* (p1081-CTX-M) and *Salmonella enterica* (pSTH21), and to a *bla*$_{CTX-M-55}$-carrying plasmid (pHN1122-1) in different parts of China (Qu et al., 2014; Lv et al., 2013), suggesting rapid dissemination of this plasmid type.

In *bla*$_{CTX-M}$-carrying IncI2 plasmids, it is notable that IS*Ecp1*-transposition units carrying four different CTX-M alleles were located in identical position between *yajA* gene and *orf16*. The intergenic sequence over here is A+T-rich (GC content 33.7%). Previous studies demonstrated that IS*Ecp1*-mediated transposition targets different variable A+T-rich sequences, and not in a site-specific manner (Cattoir et al., 2008; Poirel et al., 2005). Besides the GAAAA insertion site, the 86 bp intergenic sequence between *yajA* gene and *orf16* contains six other previously reported IS*Ecp1* targeted A+T sequences (Figure 2). Thus, the finding suggests that homologous recombination of the hybrid *bla*$_{CTX-M}$ has possibly occurred after insertion of a *bla*$_{CTX-M-15/55}$-like gene in that location, rather than vice versa. CTX-M-55 differs from CTX-M-15 by a nucleotide substitution resulting in A77V and could represent allelic variations. In the three hybrids (CTX-M-64, CTX-M-123 and CTX-M-132), the amino acid at position 77 is the same as CTX-M-15 (A77). Presence of a fragment of IncI2 fragment in the 5811 bp transposition unit carrying *bla*$_{CTX-M-123}$ in an IncI1 backbone further support the hypothesis that hybrid *bla*$_{CTX-M-123}$ originated from an IncI2 plasmid. Additional in vitro

experiments involving IncI2 plasmids are required to prove the temporal sequences of the IS*Ecp1*-transposition and homologous recombination events.

In conclusion, this study revealed widespread occurrence of the newly described hybrid CTX-M enzymes among *E. coli* isolates from a wide range of animals. Two groups of IncI1 and IncI2 plasmids harboring different hybrid CTX-M alleles were mainly involved in their dissemination. The high detection rates among isolates from food animals is concerning because rapid ongoing spread to clinical isolates in human is possible through the food chain and the contaminated environment.

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Table 1. Distribution of CTX-M subgroups among the ESBL-producing *E. coli* isolates

Source	No (%, by row) by CTX-M subgroup					
	M1	M9	M1+M9	Hybrid	None	Subtotal
Animals						
dogs	17 (42.5)	19 (47.5)	1 (2.5)	1 (2.5)	2 (5.0)	40 (100)
cats	7 (50.0)	5 (35.7)	1 (7.1)	0 (0)	1 (7.1)	14 (100)
rodents	3 (50.0)	1 (16.7)	0 (0)	1 (16.7)	1 (16.7)	6 (100)[a]
chickens	10 (26.3)	9 (23.7)	7 (18.4)	8 (21.1)	4 (10.5)	38 (100)
pigs	22 (39.3)	21 (37.5)	0 (0)	2 (3.6)	11 (19.6)	56 (100)
cattle	8 (50.0)	6 (37.5)	0 (0)	2 (12.5)	0 (0)	16 (100)
Subtotal	67 (39.4)	61 (35.9)	9 (5.3)	14 (8.2)	19 (11.2)	170 (100)
Human	23 (29.5)	53 (67.9)	1 (1.3)	0 (0)	1 (1.3)	78 (100)
Total	90 (36.3)	114 (46.0)	10 (4.0)	14 (5.6)[b]	20 (8.1)	248 (100)

[a]The six rodent isolates include five from *Rattus norvegicus* and one from *R. rattus*. The isolate tested positive for hybrod CTX-M originated from a *R. norvegicus*

[b]Including M1+hybrid (n=1, rodent), hybrid (n=13).

Table 2. Characteristics of CTX-M hybrid-carrying *E. coli* isolates and plasmids

CTX-M gene and origin	Date	Isolate	Phylogenetic group	MLST	Size (kb)	Replicon	RFLP-*Apa*LI	RFLP-*Eco*RI	Resistance in Transconjugant[c]
***bla*$_{\text{CTX-M-123}}$,**									
Chicken	2013.1	C0925A	E	ST1485	110	I1	C1	F	ESBL, Fot
Chicken	2013.2	C0964A	D	ST2732	110	I1	C2	F	ESBL, Fot
Chicken	2013.3	C0996A[a]	A	ST10	110	I1	C3	F	ESBL, Fot
Pig	2013.5	P490A	F	ST457	110	I1	C3	F	ESBL, Fot
Dog	2012.9	NTD0033A	B1	ST1196	110	I1	C3	F	ESBL, Fot
Cattle	2013.8	X0520A[b]	A	ST48	-	NT	-	-	-
***bla*$_{\text{CTX-M-64}}$**									
Chicken	2012.7	C0851A	E	ST1011	70	I2	A1	D1	ESBL
Chicken	2012.9	C0906A	E	ST1011	70	I2	A1	D1	ESBL
Chicken	2013.2	C0967A[a]	E	ST1011	70	I2	A1	D1	ESBL
Chicken	2013.3	C0992A	B1	ST224	70	I2	A2	D2	ESBL
Chicken	2013.7	C1055A	A	ST93	70	I2	A3	D1	ESBL
Rodent	2013.8	R1015A	F	ST117	70	I2	A1	D1	ESBL
***bla*$_{\text{CTX-M-132}}$**									
Pig	2012.9	P0421T[a]	A	ST3103	70	I2	B	E	ESBL
Cattle	2013.1	X0422A[b]	A	ST746	-	NT	-	-	-

MLST, multilocus sequence typing; RFLP, restriction fragment length polymorphism; NT, nontypable; -, not done

[a]The *bla*$_{\text{CTX-M}}$ carrying plasmid in the three strains were completely sequenced.

[b]No transconjugant or transformant could be obtained after multiple attempts.

[c]Resistance phenotypes involving extended-spectrum β-lactamases (ESBL), amikacin (Amk), chloramphenicol (Chl), ciprofloxacin (Cip), fosfomycin (Fot), gentamicin (Gen), cotrimoxazole (Sxt) and tetracycline (Tet).

Figure 1. Comparative analysis of linear plasmid maps for (A) three IncI1 plasmids, and (B) nine IncI2 plasmids. R64 and R721 are used as the IncI1 and IncI2 references, respectively. Grey shading denotes shared regions of homology and the similarities for between plasmids comparison are given as % coverage/% nucleotide identity. ORFs are represented by boxes and colored according to predicted gene function. White boxes indicate the plasmid scaffold regions. Yellow boxes indicated genes associated with plasmid transfer, including the *tra* locus, *trb* locus and *pil* locus. The *tra*, *trb* and *pil* genes are indicated in capital letter inside the boxes. Blue boxes indicate the genes associated with plasmid stability and maintenance. Green boxes indicate the mobile elements. Red boxes indicate the resistance genes. Orange boxes indicated the shufflon regions including the *rci* genes. Pink boxes indicated the replication-associated genes. Pink circles indicate the replication origin of transfer (*oriT*). All genes in the plasmids are shown, but the genes and regions in the maps are not drawn exactly in proportion to the length of the sequences. GenBank accession and sizes of the plasmids were as follows: pHNAH4-1 (KJ125070, 108.7 kb), pCTXM123_C0996 (KP198616, 108.4 kb), R64 (AP005147, 120.8 kb), p1081-CTXM (KJ460501, 62.2 kb), pHN1122-1 (JN797501, 62.2 kb), pSTH21 (LN623683, 62.1 kb), pHNAH46-1 (KJ020576, 62.2 kb), pCTXM64_C0967 (KP091735, 62.2 kb), pHNLDH19 (KM207012, 62.2 kb), pCTX-M132_P0421 (KP198615, 63.1 kb), pHNY2 (KF601686, 65.4 kb), R721 (AP002527, 75.6 kb).


(A)
pHNAH4-1, Anhui, 2010, chicken, E. coli
pCTXM123_C0996, Hong Kong, 2013, chicken, E. coli
R64, 1966, Salmonella typhimurium
96.9%/93.1%
blaCTX-M-123 carrying plasmids
trb locus
tra locus
pil locus
(B)
p1081-CTXM, Hangzhou, 2010, human, Shigella sonnei
pSTH21, Hong Kong, Salmonella enterica
pHN1122-1, Gunagzhou, 2007, dog E. coli
pHNAH46-1, Anhui, 2011, chicken, E. coli
pCTXM64_C0967, Hong Kong, 2013, chicken, E. coli
pHNLDH19, Guangzhou, human, E. coli
pCTXM132_P0421, Hong Kong, 2012, pig, E. coli
pHNY2, Guangzhou, chicken, E. coli
R721, E. coli
100%/98.1%
99.0%/99.8%
100%/99.6%
100%/96.7%
100%/98.1%
100%/96.7%
98.5%/90.6%
blaCTX-M-55 carrying plasmids
blaCTX-M-64 carrying plasmids
blaCTX-M-132 carrying plasmids
blaCTX-M-15 carrying plasmid
trs locus
tra locus
pil locus
trb locus

27

28

Figure 2. Schematic representation of insertion of transposition units harboring *bla*$_{CTX-M}$ in IncI1 and IncI2 plasmid scaffold. A putative 5811 bp transposition unit carrying *bla*$_{CTX-M-123}$, 112 bp IncA/C and 2715 bp IncI2 remnant fragments was inserted in the intergenic space between *ybeB* and *yedA* in two IncI1 plasmid scaffolds and was flanked by two direct repeats (DR2, TTATAC). Another 3090 bp IS*Ecp1*-transposition unit carrying *bla*$_{CTX-M-55}$ (p1081-CTXM, pHN1122-1, pSTH21), *bla*$_{CTX-M-64}$ (pHNAH46-1, pCTXM64_C0967), *bla*$_{CTX-M-132}$ (pHNLDH19, pCTXM132_P0421) or *bla*$_{CTX-M-15}$ (pHNY2) with a similar immediate genetic context was inserted in the intergenic space between *yjaA* and *orf16* in eight IncI2 plasmid scaffolds, creating two direct repeats (DR1, GAAAA). The open reading frames (ORFs) and genes are indicated by arrows and colored as in Figure 1. Direct repeat sequences are indicated in red. GenBank accession numbers of the two additional plasmids not shown in Figure 1 are: pBK15692 (KC845573) and pIFM3803 (KF787110). DR, direct repeat; IRL, left inverted repeat; IRR, right inverted repeat