Co-spread of metal and antibiotic resistance within ST3-IncH12 plasmids from *E. coli* isolates of food-producing animals

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Concerns have been raised in recent years regarding co-selection for antibiotic resistance among bacteria exposed to heavy metals, particularly copper and zinc, used as growth promoters for some livestock species. In this study, 25 IncH12 plasmids harboring *oqxAB* (20/25)/*bla\(\text{CTX-M}\) (18/25) were found with sizes ranging from ∼260 to ∼350 kb and 22 belonged to the ST3-IncH12 group. In addition to *bla\(\text{CTX-M}\)* and *oqxAB*, *pcoA-E* (5/25) and *silE-P* (5/25), as well as *aac(6')-Ib-cr* (18/25), *floxR* (16/25), *rmtB* (6/25), *qnrS1* (3/25) and *fosA3* (2/25), were also identified on these IncH12 plasmids. The plasmids carried *pco* and *sil* contributed to increasing in the MICs of CuSO\(_4\) and AgNO\(_3\). The genetic context surrounding the two operons was well conserved except some variations within the *pco* operon. The ~32 kb region containing the two operons identified in the IncH12 plasmids was also found in chromosomes of different Enterobacteriaceae species. Further, phylogenetic analysis of this structure showed that Tn7-like transposon might play an important role in cross-genus transfer of the *sil* and *pco* operons among Enterobacteriaceae. In conclusion, co-existence of the *pco* and *sil* operons, and *oqxAB/*bla\(\text{CTX-M}\)* as well as other antibiotic resistance genes on IncH12 plasmids may promote the development of multidrug-resistant bacteria.

The horizontal transfer of plasmids plays a significant role in the dissemination of antibiotic resistance genes. Plasmids in the H1 incompatibility group (IncH1) occur widely in the Enterobacteriaceae. Members of this group can carry a wide variety of resistance genes including those encoding the metallo-β-lactamase NDM-1\(^1,2\). One subgroup of IncH1, IncH12, is one of the most common incompatibility groups of plasmids in Enterobacteriaceae\(^3\). This group is frequently detected in *Salmonella enterica*, *Enterobacter cloacae*, *Klebsiella pneumonia* and *Escherichia coli* isolates from humans and chickens\(^4,6,7\), but also with a sporadic occurrence in swine\(^8\).

IncH12 plasmids have been found to carry numerous classes of resistance genes including resistance to β-lactams (*bla\(\text{CTX-M}\), *bla\(\text{CMY}\), *bla\(\text{SHV}\), *bla\(\text{TIM}\), *bla\(\text{VIM}\)*), quinolones (*oqxAB*, *qnrA1*, *qnrS1* and *qnrB2*), aminoglycosides (*armA*, *aac-Itb/aac-Ilb-cr*), amphenicols (*floR*) and fosfomycin (*fosA3*)\(^9,10\). Reports on co-spread of extended-spectrum β-lactamase (ESBLs) and plasmid-mediated quinolone resistance determinants (PMQRs) in the same plasmids have increased in the past years\(^6,11\). Our previous studies determined that IncH12 plasmids are linked to the distribution of *oqxAB-bla\(\text{CTX-M}\)* genes in *E. coli* and *Salmonella spp.*\(^10,11\). However, only a few of these cases have been documented. Fluoroquinolones such as ciprofloxacin and enrofloxacin, and cephalosporins such as ceftiofur have been widely used in veterinary medicine in China. Olaquindox, the main substrate for OqxAB, is also commonly used as a therapeutic and preventive antibiotic in pigs\(^1,2\).

In addition to genes encoding antibiotic resistance, the IncH12 plasmids also harbor a large number of metal tolerance genes. For example, R478 is the prototype of the ST1-IncH12 plasmids and has been totally sequenced\(^1\).

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It encodes efflux systems to detoxify copper \((pcoABCDRSE)\), silver \((silESRCBAP)\), arsenic \((arsCBRH)\), as well as the \(Tn1696\)-related mercury operon \((merEDACPTR)\) and tellurite resistance systems \((terZABCDEF)\) and \((terY3ZYXY1W)\). Moreover, trace elements including copper have been used as feed additives for the treatment of swine and poultry disease control and weight improvement\[16,17\].

There is increasing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance\[18\]. There is indeed experimental evidence that exposure to heavy metals (particularly copper and zinc) can induce or select for bacterial adaptations that result in decreased susceptibility to \(\beta\)-lactams\[19\]. This may occur by selection of heavy metal resistance determinants for resistance to non-antibiotic agents that are linked to genes for antibiotic resistance\[20\]. Considering that the IncHI2 plasmids may play an important role in dissemination of antibiotic and metal resistance genes, we characterized IncHI2 plasmids harboring \(oqxAB\) and/or \(bla\) in \(E. coli\) isolates from the diseased food-producing animals in China. Furthermore, the genetic context surrounding the \(pco\) and \(sil\) operons located on these IncHI2 plasmids were also investigated.

**Results**

**The prevalence of the IncHI2 plasmids.** Our initial study group contained 739 \(E. coli\) isolates from diseased animals. 405 of these isolates possessing either \(bla\) \((204)\) or \(oqxAB\) \((328)\) were selected for conjugation experiments. We were successful in obtaining 163 transconjugants harboring \(bla\) and/or \(oqxAB\), including 25 that carried IncHI2 plasmids \((25/163, 15.3\%)\). The donor strains of these 25 transconjugants were isolated from 14 ducks, 4 chickens and 7 pigs among 2004–2012 and these food-producing animals were from 15 farms (Table 1).

**Detection of antimicrobial and heavy metal resistance determinants.** Among the 25 transconjugants harboring IncHI2 plasmids, 20 carried \(oqxAB\), and 17 harbored \(bla\) \((9\%\)) while only one was positive for \(bla\). The most predominant CTX-M-encoding gene was \(bla\) \((6\%)\), followed by \(bla\) \((5\%)\) and \(bla\) \((5\%)\) (6 each). \(OqxAB\) and \(bla\) were found together in 13 transconjugants (Table 1). Other antibiotic-resistance determinants, \(aac\) \((6\%)-Ib-cr\), \(floR\), \(qnrS1\), and \(fosa3\) were co-transferred in 18, 16, 3, and 2 transconjugants, respectively. The number of transconjugants carrying \(oqxAB-aac\) \((6\%)-Ib-cr\), \(oqxAB-floR\), and \(oqxAB-aac\) \((6\%)-Ib-cr-floR\), were 15, 12, and 11, respectively. Moreover, four transconjugants carried \(oqxAB\), \(bla\) and \(fosa3\) simultaneously (Table 1). Interestingly, all of the 25 transconjugants carried a tellurite-resistance system while mercury and arsenic resistance genes were not detected. \(PcoA\) \(-D\), as well as \(sil\) genes was found in four transconjugants. Additionally, in one transconjugants \(S151T\), \(pco\) \(-E\) was observed, while \(pcoB\) was not detected. (Table 1).

**Antimicrobial susceptibility tests.** Among the 25 transconjugants harboring IncHI2 plasmids, 18 carried \(bla\) \((8\%)\) and showed a reduced susceptibility to CTX \((MIC \geq 2\mu g/mL)\). In addition, 25 and 15 transconjugants were also resistant to AMP and CIP, respectively. At least one PMQR gene was found in 25 transconjugants (except \(S100T\)). Ciprofloxacin MICs were mainly grouped into two levels including 15 non-susceptible transconjugants \((0.06–0.25\mu g/mL)\) and 9 with low resistance levels \((0.5–4\mu g/mL)\). The MICs of OQX in 20 transconjugants carrying \(oqxAB\), had 4-fold higher than that for the recipient \(E. coli\) \(C600\). All transconjugants showed increase in MICs of FLF, and 11 showed extremely high-level resistance with MICs \(\geq 256\mu g/mL\) respectively. Notably, co-transfer of extremely high-level resistance to AMK and FOS \((MICs \geq 256\mu g/mL)\) were also observed in six transconjugants harboring \(rmtB\) and two carrying \(fosa3\), respectively. None of the transconjugants were resistant to meropenem. The metal susceptibility testing showed that 5 transconjugants carrying the \(pco\) and \(sil\) genes had the MICs of CuSO\(_4\) and AgNO\(_3\), higher than that for the recipient \(E. coli\) \(C600\) \((MIC_{CuSO_4} = 12\mu M\) vs. \(8\mu M; MIC_{AgNO_3} = 0.0393 \geq 1\mu M\) vs. \(0.008\mu M\)), while in the other 20 of 25 transconjugants, the MICs of CuSO\(_4\) and AgNO\(_3\) had no change, when compared with \(E. coli\) \(C600\) (Table 1).

**Plasmids analysis.** The result of S1-PFGE revealed that all of the 25 transconjugants carried only one plasmid with size ranging from \(\sim 260\) kb to \(\sim 380\) kb, except for \(S151T\) which carried two plasmids \((\sim 260\) kb and \(\sim 100\) kb) (Table 1). Southern blot analysis confirmed that these large plasmids were members of the IncHI2 type. Moreover, a probe hybridizing to \(oqxAB\) \(/\) \(bla\) \(\sim 100\) kb) \((328)\) were selected for conjugation (Table 1). In one transconjugants \(S77\), both the \(bla\) \((9\%)\) allele and the genetic environment of the \(oqxAB\) \((IS26-oqxA-oqXB-IS26)\) were not determined. The \(oqxAB\) genes were flanked by two copies of IS26 that were located in the same orientation in 20 transconjugants harboring \(oqxAB\). To determine the stability of this structure \((IS26-oqxA-oqXB-IS26)\), inverse PCR was performed and amplicons of approximately \(1.6\) kb were obtained in all of 20 transconjugants. Sequence analysis of the amplicons further confirmed the genetic environment surrounding the \(oqxAB\) genes as obtained by PCR mapping.

**Analysis of the genetic environment of the \(oqxAB\) and \(bla\) \(\sim 100\) kb genes.** The genetic environment of the \(bla\) \((IS26-lbla\) \((16)\), while it was \(Isecp1-lbla\) \((16)\) for the \(bla\) \((9\%)\) genes (1). In one transconjugants \(S77\), both the \(bla\) \((9\%)\) allele and the genetic environment of the \(oqxAB\) \((IS26-oqxA-oqXB-IS26)\) were not determined. The \(oqxAB\) genes were flanked by two copies of IS26 that were located in the same orientation in 20 transconjugants harboring \(oqxAB\). To determine the stability of this structure \((IS26-oqxA-oqXB-IS26)\), inverse PCR was performed and amplicons of approximately \(1.6\) kb were obtained in all of 20 transconjugants. Sequence analysis of the amplicons further confirmed the genetic environment surrounding the \(oqxAB\) genes as obtained by PCR mapping.
| Strain | Source | Farm no. | Year | ESBLs | PMQRs | Co-transferred resistance genes | MICs (µg/ml)/(µM) | Plasmid |
|--------|--------|----------|------|-------|-------|---------------------------------|-------------------|---------|
| Z39    | Chicken| Farm 1   | 2004 | bla<sub>CTX-M-27</sub> | oqxAB, aac(6’)-Ib-cr | rmtB, floR | 8 0.25 8 0.008 | H12, F11 | ~350 hok-sok, pemKI, srnBC |
| Z13    | Chicken| Farm 1   | 2004 | | oqxAB | pcoA-D-E, silE-P | 0.06 0.06 12 0.03 | H12, F11 | ~350 hok-sok |
| Z31    | Chicken| Farm 1   | 2004 | | oqxAB | - | 0.06 0.06 8 0.008 | H12, F11 | ~350 hok-sok, pemKI, ccdAB |
| S7     | Pig    | Farm 2   | 2004 | bla<sub>CTX-M-9G</sub> | oqxAB, aac(6’)-Ib-cr | floR | 4 0.5 8 0.008 | H12 | ~280 |
| X2     | Duck   | Farm 3   | 2005 | bla<sub>CTX-M-45</sub> | oqxAB, aac(6’)-Ib-cr | floR | 32 1 8 0.008 | H12, N | ~280 |
| A84    | Duck   | Farm 4   | 2005 | | oqxAB | pcoA-D-E, silE-P | 0.25 0.125 12 0.06 | H12, F11 | ~280 hok-sok |
| A64    | Duck   | Farm 5   | 2007 | bla<sub>CTX-M-27</sub> | oqxAB, aac(6’)-Ib-cr | rmtB, floR | 32 0.25 8 0.008 | H12, F11 | ~350 hok-sok |
| A69    | Duck   | Farm 5   | 2007 | | oqxAB, aac(6’)-Ib-cr | rmtB, floR | 32 0.125 8 0.008 | H12, F11 | ~350 hok-sok |
| A74    | Duck   | Farm 5   | 2007 | | oqxAB, aac(6’)-Ib-cr | rmtB, floR | 32 0.5 8 0.008 | H12, F11 | ~320 hok-sok |
| A78    | Duck   | Farm 5   | 2007 | | oqxAB, aac(6’)-Ib-cr | - | 32 0.25 8 0.008 | H12, N | ~280 |
| S100   | Duck   | Farm 6   | 2007 | bla<sub>CTX-M-14</sub> | - | floR | 32 0.03 8 0.008 | H12, F11, N | ~280 hok-sok |
| S151   | Duck   | Farm 6   | 2007 | | oqxAB, aac(6’)-Ib-cr | pcoA-D-E, silE-P | 0.06 0.06 12 >1 | H12, F11 | ~260/100 hok-sok |
| P2-3   | Pig    | Farm 7   | 2008 | bla<sub>CTX-M-45</sub> | oqxAB, aac(6’)-Ib-cr | floR, fosA3 | 32 0.5 8 0.008 | H12, F11 | ~380 hok-sok, pemKI |
| P3-3   | Pig    | Farm 7   | 2008 | | oqxAB | - | 8 0.25 8 0.008 | H12, F11, F6b | ~260 |
| HAI    | Pig    | Farm 8   | 2009 | | oqxAB, aac(6’)-Ib-cr | - | 0.06 0.25 8 0.008 | H12 | ~280 |
| FS341G | Duck   | Farm 9   | 2010 | bla<sub>CTX-M-45</sub> | qmrS1, aac(6’)-Ib-cr | floR | 32 0.6 8 0.008 | H12, F11 | ~280 VagCD |
| 45-6   | Pig    | Farm 10  | 2010 | | oqxAB, aac(6’)-Ib-cr | - | floR, fosA3 | 32 0.125 8 0.008 | H12 | ~280 |
| FS271K | Duck   | Farm 9   | 2010 | bla<sub>CTX-M-45</sub> | aac(6’)-Ib-cr | floR | 32 0.125 8 0.008 | H12, N | ~280 |
| 2Y4G   | Duck   | Farm 11  | 2011 | bla<sub>CTX-M-45</sub> | oqxAB, aac(6’)-Ib-cr | floR | 2 0.25 8 0.008 | H12 | ~280 |
| 3YG7   | Duck   | Farm 11  | 2011 | | oqxAB, aac(6’)-Ib-cr | pcoA-D-E, silE-P | 0.06 0.5 12 >1 | H12 | ~280 |
| CBJ3C  | Chicken| Farm 12  | 2012 | bla<sub>CTX-M-14</sub> | oqxAB | floR, fosA3 | 16 0.6 8 0.008 | H12, N | ~350 pemKI, srnBC |
| FS8Z4C | Pig    | Farm 13  | 2012 | bla<sub>CTX-M-45</sub> | qmrS1 | floR | 32 2 8 0.008 | H12, F11 | ~280 hok-sok |
| FS1Z4S | Pig    | Farm 14  | 2012 | | oqxAB, aac(6’)-Ib-cr | - | floR | 8 0.125 8 0.008 | H12 | ~280 |
| FSTZ5G | Pig    | Farm 12  | 2012 | bla<sub>CTX-M-14</sub> | aac(6’)-Ib-cr | pcoA-D-E, silE-P | - 32 0.5 12 >1 | H12, N | ~280 |
| FS11Y5C| Duck   | Farm 15  | 2012 | bla<sub>CTX-M-45</sub> | oqxAB, aac(6’)-Ib-cr, qmrS1, aac(6’)-Ib-cr, qmrS1 | - | 64 4 8 0.008 | H12 | ~260 |

Table 1. Characteristics of the 25 E. coli isolates and transconjugants harboring IncHI2 plasmids. CTX, cefotaxime; CIP, ciprofloxacin; “-” “not detected”; “NT” “not determined”.

Analysis of the genetic environment of pco and sil genes. The regions surrounding the pco and sil genes are shown in Fig. 1, Supplementary Fig. S2 and Table S3. A Tn7-like transposon (~5.99 kb) encompassing the tnsABC genes, and a ~4.64-kb region including four ORFs (encoding hypothetical proteins), were present upstream from the sil operon, which consisted of silESRCBAP genes (~12.45 kb). That was followed by a ~1.29-kb region including two ORFs (encoding hypothetical proteins). Downstream from it, three different genetic organizations were found within the pco operon: type I, in the plasmids p3YG7T and pFS7Z5GT, a ~7.53-kb segment containing the pcoEABCDRSE genes was present; type II, in the plasmid p5151T, the pco operon was identical to that in pEC5207 (KT347600) and the pcoD and pcoR genes were deleted; type III, in the plasmids pZ13T and pA58T, the pco operon was divided into two parts, and they were not genetically linked together: in one part, downstream from pcoE, pcoA had 1348 bp deleted at the 5'-end and then was followed by an insertion sequence insL (Fig. 1); in the other part, pcoA was truncated at the 5'-end by the insertion of inpA in the reverse orientation. pcoBDCRSE was present downstream (Supplementary Fig. S1). The pco operon was then followed by a 5.69-kb region including five ORFs in these five plasmids (Fig. 1 and Supplementary Fig. S1).

To clarify the role of tnsABC~--4.64-kb region-silESRCBAP~--1.29-kb region-pcoEABCDRSE (~32kb) in spread of the sil and pco operons, the similar regions from plasmids pZ13T (tnsABC~--4.64-kb region-silESRCBAP~--1.29-kb region-pcoEΔpcoA) and p3YG7T (tnsABC~--4.64-kb region-silESRCBAP~--1.29-kb region-pcoE~--pcoR) were used for transconjugation experiments. In these experiments, the plasmids pZ13T and p3YG7T could efficiently transfer to recipients.
Figure 1. Characteristic of the genetic contexts of the pco and sil operons and linear comparison of the structures containing the two operons. The plasmid pHXY0809 (KM877269) represented an IncHI2 plasmid not carrying the sil and pco operons. Plasmids pAPEC-O1-R (BX663045), R478 (DQ517526), pSH111_227 (JN983042), and pEC5207 (KT347600) were the only four IncHI2 plasmids harbored the sil and pco operons assigned in GenBank. E. coli (CP003289), E. cloacae (CP010384), S. enterica (CP007530), C. freundii (CP012554), E. asburiae (CP0122162) represented the sequences containing the sil and pco operons and they were located on chromosomes of five different Enterobacteriaceae species. p3YG7T, pFS7Z5GT, pS151T, pZ13T, and pA84T represented the IncHI2 plasmids harbored the sil and pco operons in this study. The arrows represent the positions and transcriptional directions of the ORFs. Regions of homology are shaded in gray.

Discussion

In this study, 25 IncHI2 plasmids carrying blaCTX-M/oqxAB were found from 739 E. coli isolates from disease food-producing animals among 2004–2012. Additionally, we also found that aac(6’)-Ib-cr and floR were frequently co-transferred with blaCTX-M/oqxAB among these IncHI2 plasmids. OqxAB along with aac(6’)-Ib-cr, floR, and blaCTX-M were identified on the same transferable IncHI2 plasmids. This may be an important mechanism for dissemination of multidrug-resistance genes. Notably, blaCTX-M, oqxAB, and rmtB were the first time identified simultaneously on the IncHI2 plasmids from four transconjugants, which also showed different levels of MICs increased with CTX, CIP, and AMK as compared with recipient strain E. coli C600. The third-generation cephalosporin, fluoroquinolones, and aminoglycosides are the important front-line antibiotics. Therefore, these multidrug-resistant IncHI2 plasmids should be of great concern.

Insertion sequences ISEcp1 and IS26 are most frequently associated with blaCTX-M and oqxAB, respectively. This is consistent with our results that the blaCTX-M gene (except pS7T) and the oqxAB genes were flanked by IS26 in this study. Interestingly, inverse PCR performed on all of the oqxAB-positive transconjugants produced an amplicon and subsequent sequencing showed that the pair of intact IS26 flanking oqxAB could loop out the intervening sequence through homologous recombination. This might further accelerate oqxAB dissemination among Enterobacteriaceae. Further studies are required to explore how the diverse resistance genes, especially blaCTX-M and oqxAB, as well as rmtB, were integrated into the same IncHI2 plasmids.

IncHI2 plasmids are high molecular weight and possess multiple replications (RepHI1A and RepHI2). Additionally, the IncHI2 and IncFIB plasmids co-resident within the same cell were found to undergo plasmid fusion in the transconjugants. In this study, the majority of the plasmids contained IncHI2 were in combination with either IncFII or IncN. Plasmid may have recombined with co-resident plasmids, thereby expanding the number of replications and extending host ranges of the fused plasmids. Although the IncHI2 plasmids herein were of diverse sizes, 22 of 25 were assigned to the ST3 group by pDLST. In Europe and the USA, blaCTX-M.2 producers from both human and poultry sources have been associated with ST2-IncHI2 plasmids, while the blaCTX-M.9 producers were associated with ST1-IncHI2 plasmids. In China, ST3-IncHI2 plasmids have been found to spread fosA3 among E. coli isolates from chickens. This indicates that ST3-IncHI2 plasmids most often associated with resistance genes in E. coli from food-producing animals in China. Further, a variety of plasmid patterns were
observed by comparing the similarity of the 25 IncHI2 plasmids using RFLP analysis, although some ones showed similar XbaI digestion profiles. Considering that IncHI2 plasmids possessed a well-conserved and stable backbone3, their diversity observed herein were probably due to the deletions or acquisition of a number of resistance genes by transposons and insertion sequences24 or IS26-mediated fusion with other plasmids25.

The presence of addiction systems encoded in resistance plasmids may allow for the maintenance and dissemination of resistance genes within a given bacterial population26. However, in this study, IncHI2 plasmids were found mostly to be devoid of addiction systems which had been previously shown12. These results are not surprising because the seven addiction systems detected in this and that studies were mainly characterized in IncF, IncI1 plasmids or Salmonella virulence plasmids25. However, hipoA/B, mucA/B, relE/B, and ter determinants involved in plasmid stabilization system were observed among all of the 25 IncHI2 plasmids. This once again suggested that these genes might play a significant role in the persistence and spread of IncHI2 plasmids.

It has been known for several decades that metal- and antibiotic-resistance genes are linked, particularly on plasmids26,27. The ter determinants were found on the IncHI2 plasmids in the previous study28 and were also observed on all of the IncHI2 plasmids in this study. However, the mer and ars determinants were not found on any of the 25 IncHI2 plasmids. The prototype of the ST1–H12 group, R478, harbors the mer and ars determinants, while they are deleted in pAPEC-O1-R, the prototype of ST2–H12 plasmids3,15,29. The IncHI2 plasmids presented in this study may be genetically distinct from R478. Five of the 25 IncHI2 plasmids (20%) also harbored the pco operons downstream from the pco operon composed of pcoEABCDRSE genes in IncHI plasmids from previous reports33,34. In the current study, aTn7-like transposon carried the pco and sil genes in IncH1 plasmids from previous reports33,34. In the current study, aTn7-like transposon was also present upstream of the pco and sil operons in the five IncH1 plasmids.

Figure 2. A phylogenetic analysis of tnsABCD--4.64-kb region--silESRCBAP--1.29-kb region--pcoEABCDRSE structure among 22 reference sequences from GenBank and two sequences p21ST (KU248944) and p3YGT7(KU248945) (marked by the black triangles) in this study. The 22 reference sequences belonged to six different genera and were closely related to that in p3YGT7, with a 92–100% query coverage and an overall nucleotide identity of 99%. The GenBank accession number, the location of the sequences belonged to six different genera and were closely related to that in p3YG7T, with a 92–100% query coverage and an overall nucleotide identity of 99%.
In our previous study, we obtained the complete sequence of the IncHI2 plasmid pHXY0809 (KM877269) which carried oprAB but did not harbor the sil and pco operons. Interestingly, a linear comparison of plasmid pHXY0809 with plasmids pAPEC-O1-R, R478, pSH111-27, pEC0527, and p3YG77 (this study) revealed that the regions containing sil and pco operons appeared to be mobilized into these IncHI2 plasmids via the Tn7-based transposition (Fig. 1). We also identified a complete transposition unit flanked by 5 bp direct repeats (DR) (GTGCTT) that bounded the traABCD---4.64-kb region-silESRCABP---1.29-kb region-pcoEABCDRSE structure in the plasmid p3YG77. Furthermore, a transposition unit containing the sil and pco operons, flanked by 5 bp DR (GGTCC or GTGCTT), was also found in plasmids R478, pSH111-27, pAPEC-O1-R and pEC0527. These transposition units were all bordered by a 28 bp sequence (TGTCGAGGACAATAAGTTTGACAA) at one end, and another 28 bp sequence (AAGGATAACCTTTAATGCTTACACCA) at the other end. The two 28 bp sequences show 18-bp nucleotide identity. As Tn7 carries terminal inverted repeats of 28 bp, we speculated that the two 28 bp sequences might serve as the invert repeats of Tn7-like transposons. These results revealed that Tn7-based transpositions may play a significant role in the spread of the sil and pco operons among IncHI2 plasmids.

A chromosomal integration of Tn7-like transposons carrying the pco and sil genes was also identified in Salmonella Senftenberg. Tn7-based transposition appeared to be able to mobilize the sil and pco operons from plasmid into chromosome. Interestingly, in the IncHI2 plasmids (R478, pSH111-27, and p3YG77), the structures (masABCD---4.64-kb region-silESRCABP---1.29-kb region-pcoEABCDRSE) were highly similar to that in chromosomes of five different genera (Fig. 1). This may implicate mobilization of the sil and pco operons from plasmids into chromosomes or conversely, from the chromosomes into plasmids via Tn7-based transposition. Further, phylogenetic analysis of this structure suggested that a Tn7-like transposon was involved in cross-genus transfer of the sil and pco operons among Enterobacteriaceae of diverse origins in many countries.

Copper has been commonly used as a feed additive in animal production and as antiseptic. Silver, on the other hand, is widely used in disinfectants during production or as an animal antiseptic. There is a strong association of heavy metal micronutrients in swine feed and the occurrence and persistence of multidrug-resistant bacteria. Therefore, metal contamination may contribute to the persistence of the genetic platforms that carry metal and antibiotic resistance genes. These platforms include the IncHI2 plasmids and Tn7-like transposons which may serve to maintain and spread heavy metal-tolerant and multidrug-resistant Enterobacteriaceae.

In conclusion, we characterized 25 IncHI2 plasmids harboring blaCTX-M-opxAB from E. coli isolates from diseased farm animals in China among 2002–2012. Co-spread of blaCTX-M-opxAB with aac (6’)-Ib-cr, floR, fosA3 and rmtB, as well as the heavy metal resistance genes (pco and sil), were identified on the large and diverse ST3-IncHI2 plasmids. These IncHI2 plasmids carried the pco and sil operons also contributed to increasing in the MICs of CuSO4 and AgNO3. Further, IS26 and IS26 were found to involve in spread of blaCTX-M and opxAB, respectively. Tn7-like transposons were linked to dissemination of the sil and pco operons. This is the first report of co-existence of opxAB, blaCTX-M, and the pco and sil operons on the same plasmids. This may promote the dissemination of multidrug-resistant isolates under the metal and antibiotic selective pressure. Increased surveillance of the multidrug-resistant IncHI2 plasmids in E. coli food-producing animals is urgently needed.

Materials and Methods

Bacterial strains. A total of non-duplicate 739 E. coli strains were isolated from viscera or feces samples from diseased food-producing animals, including ducks (203), chickens (110), geese (31) and pigs (395) between 2002 and 2012 as described previously. The samples were recovered from more than 80 livestock farms throughout Guangdong province. E. coli isolates carrying the blaCTX-M and/or opxAB genes (405/739) were selected in conjugation experiments by the broth-mating method using E. coli C600 (streptomycin-resistant; MIC > 2000 μg/mL) as the recipient. The transconjugants were selected on MacConkey agar plates supplemented with streptomycin (500–1000 μg/mL) and cefotaxime (2 mg/L) or olaquindox (32–64 mg/L). The plasmids isolated from the transconjugants harboring blaCTX-M and/or opxAB were further characterized by PCR-based replicon typing (PBRT) using PCR amplification/sequencing with IncHI2 primers as previously described.

Antimicrobial susceptibility tests. For all of the transconjugants harboring IncHI2 plasmids, MICs of ampicillin (AMP), cefoxitin (FOX), cefotetracillin (CIF), cefotaxime (CTX), amikacin (AMK), gentamicin (GEN), chloramphenicol (CHL), florfenicol (FFP), doxycycline (DOX), nalidixic acids (NAL), ciprofloxacin (CIP), olaquindox (OQX), sulfamethoxazole/trimethoprim (SXT), meropenem (MEO) were determined by the agar dilution method in Mueller–Hinton agar containing 25 μg/mL glucose 6-phosphate, according to guideline M100–S20 of the CLSI. MICs of fosfomycin (FOS) was determined by the agar dilution method on Mueller–Hinton agar containing 25 μg/mL glucose 6-phosphate, according to guideline M100–S20 of the CLSI. The breakpoints for each antimicrobial were used as recommended by the CLSI (M100–S25) or CLSI (Vet01–A4/Vet01–S2) with some modification. Briefly, the transconjugants harboring IncHI2 plasmids were incubated in Mueller–Hinton broth with serial dilutions of CuSO4 [0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 32, and 36 μM, adjusted to pH 7.2] and AgNO3 [0.0004, 0.0008, 0.0015, 0.03, 0.06, 0.08, 0.125, 0.16, 0.25, 0.32, 0.5, 1.0 mM, adjusted to pH 7.4]. E. coli C600 was used as a reference strain.

Detection of antimicrobial and heavy metal resistance determinants. ESBL-encoding genes (blaTEM, blaCMY, blaSHV, blaOXA-51, blaOXA-23, blaOXA-24, and blaOXA-26), pAmpCs-encoding genes (blaOXY), PMQR genes (qnrA, qnrB, qnrS, aac(6’)-Ib-cr, qepA, oprA, and oqxAB), exogenously acquired 16S rRNA methyltransferase (16S-RMTase) genes (rmtB and armA), fosfomycin resistance genes (fosa3, fosA, and fosC2) and the florfenicol resistance gene (floR) were detected among all of the transconjugants harboring IncHI2 plasmids by PCR amplification using primers published previously. Metal resistance determinants, including terD, terF, terX and terY3 (confering resistance to tellurium), merA and merC (confering resistance to mercury), arsB and arsH
Plasmids analysis. Plasmids analysis was carried out in the transconjugants harboring IncH12 plasmids by DNA linearization with S1 nuclease followed by PFGE analysis26. *Salmonella enterica* serotype Braenderup H9812 standards and Lambda Ladder PFG marker (NEB, Biolabs) were used as size markers. Southern blotting was carried out on S1-PFGE gels with digoxigenin-labelled probes specific for the IncH12 replicon, *pcoA*, *pcoB*, *pcoC*, *pcoD*, *pcoE*, *silA*, and *silE*. Incompatibility (Inc) groups were assigned by PBR7 of the transconjugants40. Plasmid double-locus sequence typing (pDLST) for IncH12 plasmids was performed as previously described31. The IncH12 plasmids were further analyzed by restriction fragment length polymorphism (RFLP) using XbaI as the restriction enzymes (TaKaRa Biotechnology, Dalian, China). Comparison of RFLP patterns was performed with BioNumerics36, v6.6 (Applied Maths, Ghent, Belgium). Dendrograms were generated with the Dice similarity coefficient (1.5% optimization and 1.5% tolerance) using the unweighted pair group method with arithmetic mean. RFLP types were defined with ≥75% similarity between clusters. Additionally, to further understand the successful dissemination of the IncH12 plasmids, plasmid addiction systems were determined28 and another three genes *hipA*, *mucB* and *relE* involving in plasmid stabilization system were also detected (Table S1).

Analysis of the genetic environment of resistance genes. The genetic context surrounding *oqxAB* and *blaCTX-M* on the IncH12 plasmids were investigated by PCR mapping, inverse PCR and sequencing. The primers used to determine the regions upstream and downstream of the *bla* genes were designed using the software “primer3” (http://frodo.wi.mit.edu/). Comparison of RFLP patterns was performed with BioNumerics36, v6.6 (Applied Maths, Ghent, Belgium). Dendrograms were generated with the Dice similarity coefficient (1.5% optimization and 1.5% tolerance) using the unweighted pair group method with arithmetic mean. RFLP types were defined with ≥75% similarity between clusters. Additionally, to further understand the successful dissemination of the IncH12 plasmids, plasmid addiction systems were determined28 and another three genes *hipA*, *mucB* and *relE* involving in plasmid stabilization system were also detected (Table S1).

Nucleotide Sequence Accession Numbers. The two partial nucleotide sequences of plasmid pZ13T containing the *sil* operon and the *pcoBCDRSE* genes have been deposited into GenBank under accession numbers KU248944 and KU248943, respectively. The partial nucleotide sequences of plasmid p3YG77 containing the *sil* and *pco* operons has also been deposited into GenBank under accession numbers KU248945.

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**Author Contributions**

L.F. performed experiments, analyzed the data and wrote the manuscript; X.L. and S.L. performed experiments; L.L. edited the manuscript; J.S. designed the experiments, analyzed the data, and edited the manuscript. X.L. and Y.L. coordinated the whole project.

**Additional Information**

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