SCIENTIFIC REPORTS

OPEN

Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals

Liangxing Fang[1,2], Xingping Li[1,2], Liang Li[1,2], Shumin Li[1,2], Xiaoping Liao[1,2], Jian Sun[1,2] & Yahong Liu[1,2,3]

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 IncHI2 plasmids harboring *oqxAB* (20/25)/*bla*$_{CTX-M}$ (18/25) were found with sizes ranging from ~260 to ~350 kb and 22 belonged to the ST3-IncHI2 group. In addition to *bla*$_{CTX-M}$ and *oqxAB*, *pcoA-E* (5/25) and *silE-P* (5/25), as well as *aac(6′)-Ib-cr* (18/25), *floR* (16/25), *rmtB* (6/25), *qnrS1*(3/25) and *fosA3* (2/25), were also identified on these IncHI2 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 IncHI2 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*$_{CTX-M}$ as well as other antibiotic resistance genes on IncHI2 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 HI incompatibility group (IncHI) 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 IncHI, IncHI2, 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], but also with a sporadic occurrence in swine[7,8].

IncHI2 plasmids have been found to carry numerous classes of resistance genes including resistance to β-lactams (*bla*$_{CTX-M}$, *bla*$_{CMY}$, *bla*$_{SHV}$, *bla*$_{IMP}$, *bla*$_{VIM}$), quinolones (*oqxAB*, *qnrA1*, *qnrS1* and *qnrB2*), aminoglycosides (*armA*, *aac-Ib/aac-Ib-cr*), amphenicols (*floR*) and fosfomycin (*fosA3*)[3,9–12]. 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[6,13]. Our previous studies determined that IncHI2 plasmids are linked to the distribution of *oqxAB-bla*$_{CTX-M}$ 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[14].

In addition to genes encoding antibiotic resistance, the IncHI2 plasmids also harbor a large number of metal tolerance genes. For example, R478 is the prototype of the ST1-IncHI2 plasmids and has been totally sequenced[15].

[1]National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou, China. [2]Laboratory of Veterinary Pharmacology, College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, P. R. China. [3]Jiangsu Co-Innovation Centre for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu, the People's Republic of China. Correspondence and requests for materials should be addressed to J.S. (email: jiansun@scau.edu.cn) or Y.L. (email: lyh@scau.edu.cn)

It encodes efflux systems to detoxify copper (*pcoABCDRSE*), silver (*silESRCBAP*), arsenic (ars*CBRH*), as well as the Tn1696-related mercury operon (*merEDACPTR*) and tellurite resistance systems (*terZABCDEF* and *terY3Y2XY1W*). 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 β-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_{CTX-M} 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_{CTX-M} (204) or *oqxAB* (328) were selected for conjugation experiments. We were successful in obtaining 163 transconjugants harboring bla_{CTX-M} and/or *oqxAB*, including 25 that carried IncHI2 plasmids (25/163 total, 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 $bla_{CTX-M-9G}$, while only one was positive for $bla_{CTX-M-1G}$. The most predominant CTX-M-encoding gene was $bla_{CTX-M-14}$ (6), followed by $bla_{CTX-M-27}$ and $bla_{CTX-M-65}$ (5 each). *OqxAB* and bla_{CTX-M} 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*, $bla_{CTX-M-9G}$ and *rmtB* 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-E*, as well as sil*E-P* genes was found in four transconjugants. Additionally, in one transconjugants S151T, *pcoA-E* was observed, while *pcoD* was not detected. (Table 1).

Antimicrobial susceptibility tests.
Among the 25 transconjugants harboring IncHI2 plasmids, 18 carried bla_{CTX-M} 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 CIF, 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 μg/mL) and 9 with low resistance levels (0.5–4 μg/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 $\geq 256\mu g/mL$. 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 mM vs. 8 mM; MIC$_{AgNO_3}$ = 0.03~>1 mM vs. 0.008 mM), 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 ~260 kb to ~380 kb, except for S151T which carried two plasmids (~260 kb and ~100 kb) (Table 1). Southern blot analysis confirmed that these large plasmids were members of the IncHI2 type. Furthermore, a probe hybridizing to *oqxB*/$bla_{CTX-M-9G}$/$bla_{CTX-M-1G}$/*rmtB*/*pcoA*/*silE* also confirmed that these genes were located on the IncHI2 plasmids. Interestingly, 16 of 25 (except pS151T) were fused plasmids. The most prevalent combination was IncHI2 in combination with IncFII (10) and followed by IncN (6) (Table 1). Using pDLST analysis, 22 IncHI2 plasmids were assigned to ST3 and only one to ST1 (pZ13T). Two IncHI2 plasmids were not typeable due to a failure to detect the smr0199 loci (pA84T and pS100T). RFLP analysis of plasmid DNA from the transconjugants harboring IncHI2 plasmids using *XbaI* demonstrated that 21 of 25 could be divided into eleven groups (designated A to K) ($\geq 75\%$ similarity) (Table 1).

The *hipA*, *mucB* and *relE* genes involved in plasmid stabilization were found in all of the 25 IncHI2 plasmids. However, the seven addiction systems tested in this study were completely lacking in eight plasmids containing only the IncHI2 replicon, as well as another four fused plasmids. Furthermore, no more than three addiction systems were detected among all of the 25 IncHI2 plasmids.

Analysis of the genetic environment of the *oqxAB* and *bla$_{CTX-M}$* genes.
The genetic environment of the $bla_{CTX-M-9G}$ genes was IS*Ecp1*-$bla_{CTX-M-9G}$-IS*903* (16), while it was IS*Ecp1*-$bla_{CTX-M-1G}$-*orf477* for the $bla_{CTX-M-1G}$ genes (1). In one transconjugants S7T, both the $bla_{CTX-M-9G}$ allele and the genetic environment of the $bla_{CTX-M-9G}$ gene 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	Co-transferred resistance genes				MICs (ug/ml)/(mM)				Plasmid			X-baI RFLP
				ESBLs	PMQRs	Metal resistance genes	Other genes	CTX	CIP	CuSO4	AgNO3	Replicon types	Size (kb)	Addiction system	
Z39	Chicken	Farm 1	2004	bla$_{CTX-M-27}$	oqxAB, aac(6')-Ib-cr	–	rmtB, floR	8	0.25	8	0.008	HI2, FII	~350	hok-sok, pemKI, srnBC	G3
Z13	Chicken	Farm 1	2004	–	oqxAB	pcoA-D-E, silE-P	rmtB	0.06	0.06	12	0.03	HI2, FII	~350	hok-sok	NT
Z31	Chicken	Farm 1	2004	–	oqxAB	–	–	0.06	0.06	8	0.008	HI2, FII	~350	hok-sok, srnBC, ccdAB	F4
S7	Pig	Farm 2	2004	bla$_{CTX-M-9G}$	oqxAB, aac(6')-Ib-cr	–	floR	4	0.5	8	0.008	HI2	~280	no	I
X2	Duck	Farm 3	2005	bla$_{CTX-M-65}$	oqxAB, aac(6')-Ib-cr	–	floR	32	1	8	0.008	HI2, N	~280	no	C
A84	Duck	Farm 4	2005	–	oqxAB	pcoA-D-E, silE-P	rmtB	0.25	0.125	12	0.06	HI2, FII	~280	hok-sok	G2
A64	Duck	Farm 5	2007	bla$_{CTX-M-27}$	oqxAB, aac(6')-Ib-cr	–	rmtB, floR	32	0.25	8	0.008	HI2, FII	~350	hok-sok	NT
A69	Duck	Farm 5	2007	bla$_{CTX-M-27}$	oqxAB, aac(6')-Ib-cr	–	rmtB, floR	32	0.125	8	0.008	HI2, FII	~350	hok-sok	E
A74	Duck	Farm 5	2007	bla$_{CTX-M-27}$	oqxAB, aac(6')-Ib-cr	–	rmtB, floR	32	0.5	8	0.008	HI2, FII	~320	hok-sok	NT
A78	Duck	Farm 5	2007	bla$_{CTX-M-27}$	oqxAB, aac(6')-Ib-cr	–	–	32	0.25	8	0.008	HI2, N	~280	no	G1
S100	Duck	Farm 6	2007	bla$_{CTX-M-14}$	–	–	floR	32	0.03	8	0.008	HI2, FII, N	~280	hok-sok	H
S151	Duck	Farm 6	2007	–	oqxAB, aac(6')-Ib-cr	pcoA-E, silE-P	–	0.06	0.06	12	>1	HI2, FII	~260/100	hok-sok	D2
P2-3	Pig	Farm 7	2008	bla$_{CTX-M-65}$	oqxAB, aac(6')-Ib-cr	–	floR, fosA3	32	0.5	8	0.008	HI2, FIB	~380	hok-sok, pemKI	F2
P3-3	Pig	Farm 7	2008	–	oqxAB	–	–	8	0.25	8	0.008	HI2, FII, FIB	~260	no	F1
HAI	Pig	Farm 8	2009	–	oqxAB, aac(6')-Ib-cr	–	–	0.06	0.25	8	0.008	HI2	~280	no	K2
FS341G	Duck	Farm 9	2010	bla$_{CTX-M-65}$	qnrS1, aac(6')-Ib-cr	–	floR	32	0.5	8	0.008	HI2, N	~280	VagCD	A
45-6	Pig	Farm 10	2010	bla$_{CTX-M-14}$	oqxAB, aac(6')-Ib-cr	–	floR	32	0.125	8	0.008	HI2	~280	no	J2
FS271X	Duck	Farm 9	2010	bla$_{CTX-M-65}$	aac(6')-Ib-cr	–	floR	32	0.125	8	0.008	HI2, N	~280	no	J3
2Y4G	Duck	Farm 11	2011	bla$_{CTX-M-14}$	oqxAB, aac(6')-Ib-cr	–	floR	2	0.25	8	0.008	HI2	~280	no	B
3YG7	Duck	Farm 11	2011	–	oqxAB, aac(6')-Ib-cr	pcoA-D-E, silE-P	floR	0.06	0.5	12	>1	HI2	~280	no	D3
CBJ3C	Chicken	Farm 12	2012	bla$_{CTX-M-14}$	oqxAB	–	floR, foSA3	16	0.06	8	0.008	HI2, N	~350	PemKI, srnBC	NT
FS8Z4C	Pig	Farm 13	2012	bla$_{CTX-M-65}$	qnrS1	–	floR	32	2	8	0.008	HI2, FII	~280	hok-sok	F3
FS1Z4S	Pig	Farm 14	2012	bla$_{CTX-M-14}$	oqxAB, aac(6')-Ib-cr	–	floR	8	0.125	8	0.008	HI2	~280	no	K1
FS7Z5G	Pig	Farm 13	2012	bla$_{CTX-M-14}$	aac(6')-Ib-cr	pcoA-D-E, silE-P	–	32	0.5	12	>1	HI2,	~280	no	D1
FS11Y5C	Duck	Farm 15	2012	bla$_{CTX-M-55}$	oqxAB, aac(6')-Ib-cr, qnrS1 aac(6')-Ib-cr qnrS1	–	–	64	4	8	0.008	HI2	~260	no	J1

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 *tnsABCD* 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 pS151T, 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 pA84T, 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 3′-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 *tnpA* in the reverse orientation, and *pcoBCDRSE* 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 *tnsABCD*-~4.64-kb region-*silESRCBAP*-~1.29-kb region-*pcoEABCDRSE* (~32 kb) in spread of the *sil* and *pco* operons, the similar regions from plasmids pZ13T (*tnsABCD*-~4.64-kb region-*silESRCBAP*-~1.29-kb region-*pcoE*-Δ*pcoA*) and p3YG7 (*tnsABCD*-~4.64-kb
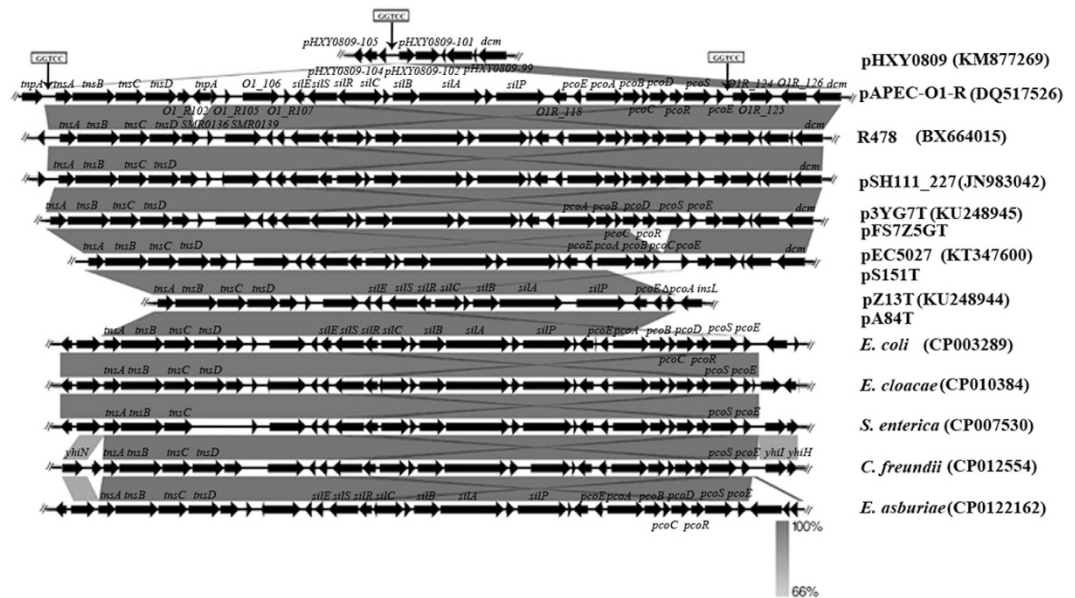
Figure 1. Characteristic of the genetic contexts of the *pco* and *sil* operons and linear comparison of the structures containing the *two* operons. The plasmid pHXY0809 (KM877269) represented an IncHI2 plasmid not carrying the *sil* and *pco* operons. Plasmids pAPEC-O1-R (BX663045), R478 (DQ517526), 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.

region-*silESRCBAP*-~1.29-kb region-*pcoEABCDRSE*) were aligned with another 22 reference sequences downloaded from GenBank's nucleotide database. The 22 reference sequences were closely related to that in p3YG7T, with a 92–100% query coverage and 99% overall nucleotide identity. A phylogenetic tree suggested that these regions were fairly conserved among the 24 selected sequences from chromosomes DNA or plasmids from isolates of six Enterobacteriaceae species. These isolates were recovered from six countries, and diverse origins including humans, animals and environment (Fig. 2).

Discussion

In this study, 25 IncHI2 plasmids carrying $bla_{CTX-M}/oqxAB$ 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 $bla_{CTX-M}/oqxAB$ among these IncHI2 plasmids. *OqxAB* along with *aac* (6′)-*Ib-cr*, *floR*, and bla_{CTX-M} were identified on the same transferable IncHI2 plasmids[10]. This may be an important mechanism for dissemination of multidrug-resistance genes. Notably, bla_{CTX-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 IS*Ecp1* and IS*26* are most frequently associated with bla_{CTX-M} and *oqxAB*, respectively[11,21]. This is consistent with our results that IS*Ecp1* was upstream of the bla_{CTX-M} gene (except pS7T) and the *oqxAB* genes were flanked by IS*26* 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 IS*26* 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 bla_{CTX-M} and *oqxAB*, as well as *rmtB*, were integrated into the same IncHI2 plasmids.

IncHI2 plasmids are high molecular weight and possess multiple replicons (RepHI1A and RepHI2)[15]. Additionally, the IncHI2 and IncFIB plasmids co-resident within the same cell were found to undergo plasmid fusion in the transconjugants[22]. 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[23], thereby expanding the number of replicons 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, $bla_{CTX-M-2}$ producers from both human and poultry sources have been associated with ST2-IncHI2 plasmids, while the $bla_{CTX-M-9}$ producers were associated with ST1-IncHI2 plasmids[4]. In China, ST3-IncHI2 plasmids have been found to spread *fosA3* among *E. coli* isolates from chickens[12]. 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
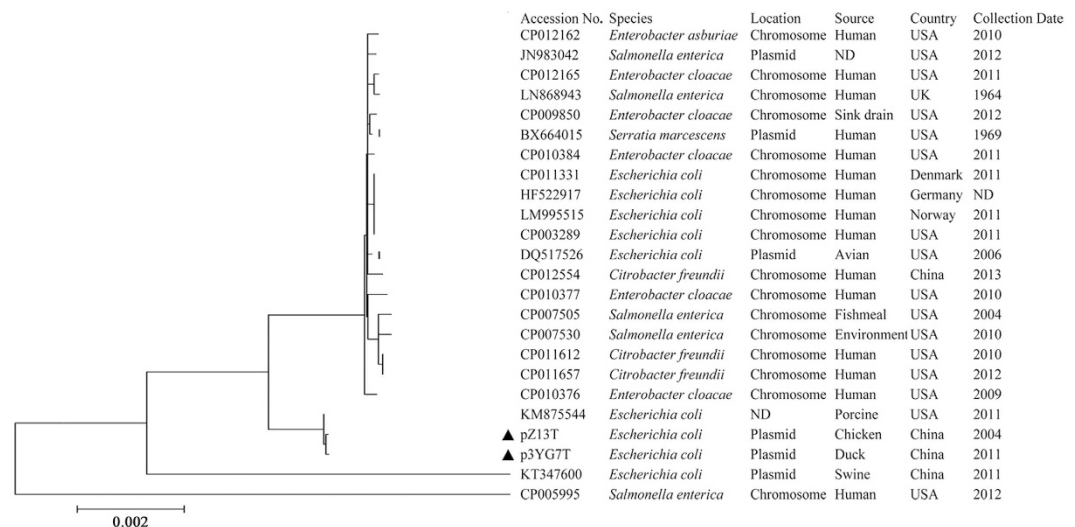
Accession No.	Species	Location	Source	Country	Collection Date
CP012162	*Enterobacter asburiae*	Chromosome	Human	USA	2010
JN983042	*Salmonella enterica*	Plasmid	ND	USA	2012
CP012165	*Enterobacter cloacae*	Chromosome	Human	USA	2011
LN868943	*Salmonella enterica*	Chromosome	Human	UK	1964
CP009850	*Enterobacter cloacae*	Chromosome	Sink drain	USA	2012
BX664015	*Serratia marcescens*	Plasmid	Human	USA	1969
CP010384	*Enterobacter cloacae*	Chromosome	Human	USA	2011
CP011331	*Escherichia coli*	Chromosome	Human	Denmark	2011
HF522917	*Escherichia coli*	Chromosome	Human	Germany	ND
LM995515	*Escherichia coli*	Chromosome	Human	Norway	2011
CP003289	*Escherichia coli*	Chromosome	Human	USA	2011
DQ517526	*Escherichia coli*	Plasmid	Avian	USA	2006
CP012554	*Citrobacter freundii*	Chromosome	Human	China	2013
CP010377	*Enterobacter cloacae*	Chromosome	Human	USA	2010
CP007505	*Salmonella enterica*	Chromosome	Fishmeal	USA	2004
CP007530	*Salmonella enterica*	Chromosome	Environment	USA	2010
CP011612	*Citrobacter freundii*	Chromosome	Human	USA	2010
CP011657	*Citrobacter freundii*	Chromosome	Human	USA	2012
CP010376	*Enterobacter cloacae*	Chromosome	Human	USA	2009
KM875544	*Escherichia coli*	ND	Porcine	USA	2011
▲ pZ13T	*Escherichia coli*	Plasmid	Chicken	China	2004
▲ p3YG7T	*Escherichia coli*	Plasmid	Duck	China	2011
KT347600	*Escherichia coli*	Plasmid	Swine	China	2011
CP005995	*Salmonella enterica*	Chromosome	Human	USA	2012

0.002

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 pZ13T (KU248944) and p3YG7T(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 p3YG7T, with a 92–100% query coverage and an overall nucleotide identity of 99%. The GenBank accession number, the location of the *sil* and *pco* operons of each sequence and the host species, the sources, the locations of recovery and the collection dates of strains of each sequence are shown. The phylogenetic tree is constructed using MEGA 5.05 software.

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 backbones[3], their diversity observed herein were probably due to the deletions or acquisition of a number of resistance genes by transposons and insertion sequences[24] or IS26-mediated fusion with other plasmids[23].

The presence of addiction systems encoded in resistance plasmids may allow for the maintenance and dissemination of resistance genes within a given bacterial population[25]. However, in this study, IncHI2 plasmids were found mostly to be devoid of addiction systems which had been previously shown[12]. 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 plasmids[25]. However, *hipA/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 plasmids[26,27]. The *ter* determinants were found on the IncHI2 plasmids in the previous study[28] 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-HI2 group, R478, harbors the *mer and ars* determinants, while they are deleted in pAPEC-O1-R, the prototype of ST2-HI2 plasmids[3,15,29]. The IncHI2 plasmids presented in this study may be genetically distinct from R478. Five of the 25 IncHI2 plasmids (20%) also harbored the *pco* and *sil* genes, simultaneously. The metal susceptibility testing showed that they also contributed to increasing in the MICs of $CuSO_4$ and $AgNO_3$ when compared with the recipient *E. coli* C600 and other transconjugants not carried the *pco* and *sil* genes. Surprisingly, there were some differences between the MICs of $AgNO_3$, but the MICs of $CuSO_4$ were identical in the five transconjugants carrying *pco* and *sil* genes.

We further analyzed the genetic background surrounding the *pco* and *sil* genes. The results indicated that the ~24-kb structures (*tnsABCD*-~4.64-kbregion-*silESRCBAP*-~1.29-kb region) and the 5.69-kb regions including five ORFs downstream from the *pco* operons were well conserved in the five plasmids in this study and another four IncHI2 plasmids (R478 (DQ517526), pSH111-27 (JN983042), pAPEC-O1-R (BX663045) and pEC5027 (KT347600)) from GenBank (Fig. 1). The *silESRCBAP* genes constituted the complete *sil* operon and shared 95~96.3% identity to that of plasmid pMG101 (AF067954) (Fig. S3A) which played a function role in conferring to silver resistance[30]. However, there was variability within the *pco* operon. In the plasmids p3YG7T and pFS7Z5GT, the complete *pco* operon composed of *pcoEABCDRSE* genes, was 99.4% identical to that of the plasmid PRJ1004 (X83541) (Fig. S3B) which facilitates copper efflux[31]. However, in another three IncHI2 plasmids pS151T, pZ13T and pA84T, the *pco* operons were disrupted even though they also shared high similarities with that of plasmid PRJ1004 (Fig. S3B). The deletion of the *pcoD* and *pcoR* genes was also observed in the IncHI2 plasmid pEC5027 carrying bla_{CMY-2} in our previous report[32]. It has been demonstrated that mutations in each of the *pcoABCD* genes on the plasmid pRJ1004 and the *silECBA* genes on plasmid pMG101 lead to complete loss of copper and silver resistance, respectively[31,33]. Thus, the reason for the inconsistency between the genetic contexts of the *pco* and *sil* operons and the MICs of $CuSO_4$ and $AgNO_3$ observed in the five transconjugants harboring *pco* and *sil* is unknown. This remains to be elucidated through further studies.

A Tn7-like transposon carried the *pco* and *sil* genes in IncHI plasmids from previous reports[33,34]. In the current study, aTn7-like transposon was also present upstream of the *pco* and *sil* operons in the five IncHI2 plasmids.

In our previous study, we obtained the complete sequence of the IncHI2 plasmid pHXY0809 (KM877269) which carried *oqxAB* 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 p3YG7T (this study) revealed that the regions containing *sil* and *pco* operons appeared to be mobilized into these IncHI2 plasmids via the Tn7-based transpositions (Fig. 1). We also identified a complete transposition unit flanked by 5 bp direct repeats (DR) (GTCCT) that bounded the *tnsABCD*-~4.64-kb region-*silESRCBAP*-~1.29-kb region-*pcoEABCDRSE* structure in the plasmid p3YG7T. Furthermore, a transposition unit containing the *sil* and *pco* operons, flanked by 5-bp DR (GGTCC or GTCCT), was also found in plasmids R478, pSH111-27, pAPEC-O1-R and pEC0527. These transposition units were all bordered by a 28 bp sequence (TGTCCGAGGACAATAAAGTTGTACACAA) at one end, and another 28 bp sequence (AAGGATACAACTTTAATGTCTCTACACA) at the other end. The two 28 bp sequences show 18-bp nucleotide identity. As Tn7 carries terminal inverted repeats of 28 bp[35], 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[34]. Tn7-based transposition appeared to be able to mobilize the *sil* and *pco* operons from plasmid into chromosome[33]. Interestingly, in the IncHI2 plasmids (R478, pSH111-27, and p3YG7T), the structures (*tnsABCD*-~4.64-kb region-*silESRCBAP*-~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 growth promotion as described above. Silver, on the other hand, is widely used in disinfectants during production or as animal antiseptics[36,37]. There is a strong association of heavy metal micronutrients in swine feed and the occurrence and persistence of multidrug-resistant bacteria[38]. 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 bla_{CTX-M}/*oqxAB* from *E. coli* isolates from diseased farm animals in China among 2002–2012. Co-spread of bla_{CTX-M}/*oqxAB* 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 $CuSO_4$ and $AgNO_3$. Further, IS*Ecp1* and IS*26* were found to involve in spread of bla_{CTX-M} and *oqxAB*, respectively. Tn7-like transposons were linked to dissemination of the *sil* and *pco* operons. This is the first report of co-existence of *oqxAB*, bla_{CTX-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[10,39]. The samples were recovered from more than 80 livestock farms throughout Guangdong province. *E. coli* isolates carrying the bla_{CTX-M} and/or *oqxAB* 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 bla_{CTX-M} and/or *oqxAB* were further characterized by PCR-based replicon typing (PBRT) using PCR amplification/sequencing with IncHI2 primers as previously described[40].

Antimicrobial susceptibility tests. For all of the transconjugants harboring IncHI2 plasmids, MICs of ampicillin (AMP), cefoxitin (FOX), ceftiofur (CIF), cefotaxime (CTX), amikacin (AMK), gentamicin (GEN), chloramphenicol (CHL), florfenicol (FLF), doxycycline (DOX), nalidixic acids (NAL), ciprofloxacin (CIP), olaquindox (OQX), sulfamethoxazole/trimethoprim (SXT), meropenem (MEO) were determined by the agar dilution method following the guidelines of Clinical and Laboratory Standards institute (CLSI). MIC 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)[41,42]. *E. coli* ATCC 25922 was used as a quality control strain. MICs of $AgNO_3$ and $CuSO_4$ were determined by broth microdilution method in an aerobic atmosphere as previously described[33], with some modification. Briefly, the transconjugants harboring IncHI2 plasmids were incubated in Mueller-Hinton broth with serial dilutions of $CuSO_4$ (0.25, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 32 and 36 mM, adjusted to pH 7.2) and $AgNO_3$ (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 (bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1G}$, $bla_{CTX-M-9G}$, $bla_{CTX-M-2G}$, and bla_{CMY-2}), pAmpCs-encoding genes (bla_{CMY-2}), PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac-(6′)-Ib-cr*, *qepA* and *oqxA* and *oqxB*), 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[9,10,12,43,44]. Metal resistance determinants, including *terD*, *terF*, *terX* and *terY3* (conferring resistance to tellurium), *merA* and *merC* (conferring resistance to mercury), *arsB* and *arsH*

(conferring resistance to arsenic), *pcoA*, *pcoD* and *pcoE* (conferring resistance to copper), *silE* and *silP* (conferring resistance to silver) were also detected among these transconjugants by PCR amplification (Table S1).

Plasmids analysis.   Plasmids analysis was carried out in the transconjugants harboring IncHI2 plasmids by DNA linearization with S1 nuclease followed by PFGE analysis[45]. *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 IncHI2 replicon, *oqxB, bla*$_{CTX-M-9G}$, *bla*$_{CTX-M-1G}$, *rmtB*, *pcoA* and *silE*. Incompatibility (Inc) groups were assigned by PBRT of the transconjugants[40]. Plasmid double-locus sequence typing (pDLST) for IncHI2 plasmids was performed as previously described[3]. The IncHI2 plasmids were further analyzed by restriction fragment length polymorphism (RFLP) using *Xba*I as the restriction enzymes (TaKaRa Biotechnology, Dalian, China). Comparison of RFLP patterns was performed with BioNumerics®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 IncHI2 plasmids, plasmid addiction systems were determined[25] 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 *bla*$_{CTX-M}$ on the IncHI2 plasmids were investigated by PCR mapping, inverse PCR and sequencing. The primers used to determine the regions upstream and downstream of the *oqxAB* and *bla*$_{CTX-M}$ genes are listed in Table S1. The genetic contexts of *pco* and *sil* genes on the IncHI2 plasmids were also explored by PCR mapping and primer walking. The region containing the *pco* and *sil* genes in plasmids pEC5207 (KT347600) was using as the reference sequence (Supplementary Table S2).

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 p3YG7T containing the *sil* and *pco* operons has also been deposited into GenBank under accession numbers KU248945.

References

1. Villa, L., Poirel, L., Nordmann, P., Carta, C. & Carattoli, A. Complete sequencing of an IncH plasmid carrying the *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$ and *qnrB1* genes. *J Antimicrob Chemother*. **67**, 1645–1650 (2012).
2. Dolejska, M., Villa, L., Poirel, L., Nordmann, P. & Carattoli, A. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. *J Antimicrob Chemother*. **68**, 34–39 (2013).
3. Garcia-Fernandez, A. & Carattoli, A. Plasmid double locus sequence typing for IncHI2 plasmids, a subtyping scheme for the characterization of IncHI2 plasmids carrying extended-spectrum beta-lactamase and quinolone resistance genes. *J Antimicrob Chemother*. **65**, 1155–1161 (2010).
4. Garcia Fernandez, A. *et al.* Comparative analysis of IncHI2 plasmids carrying *bla*$_{CTX-M-2}$ or *bla*$_{CTX-M-9}$ from *Escherichia coli* and *Salmonella enterica* strains isolated from poultry and humans. *Antimicrob Agents Chemother*. **51**, 4177–4180 (2007).
5. Villa, J. *et al.* Multiclonal spread of VIM-1-producing *Enterobacter cloacae* isolates associated with In624 and In488 integrons located in an IncHI2 plasmid. *Int J Antimicrob Agents*. **43**, 451–455 (2014).
6. Miro, E. *et al.* Spread of plasmids containing the *bla*(VIM-1) and *bla*(CTX-M) genes and the qnr determinant in *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* isolates. *J Antimicrob Chemother*. **65**, 661–665 (2010).
7. Fischer J Fau - Rodriguez, I. *et al.* *Escherichia coli* producing VIM-1 carbapenemase isolated on a pig farm. *J Antimicrob Chemother*. **67**, 1793–1795 (2012).
8. Fischer J Fau – Rodriguez, I. *et al.* *Salmonella enterica* subsp. enterica producing VIM-1 carbapenemase isolated from livestock farms. *J Antimicrob Chemother*. **68**, 478–480 (2013).
9. Fang, L. X. *et al.* Dissemination of the chromosomally encoded CMY-2 cephalosporinase gene in *Escherichia coli* isolated from animals. *Int J Antimicrob Agents*. **46**, 209–213 (2015).
10. Liu, B. T. *et al.* Dissemination and characterization of plasmids carrying *oqxAB-bla*$_{CTX-M}$ genes in *Escherichia coli* isolates from food-producing animals. *PloS one*. **8**, e73947 (2013).
11. Li, L. *et al.* Spread of *oqxAB* in *Salmonella enterica* serotype Typhimurium predominantly by IncHI2 plasmids. *J Antimicrob Chemother*. **68**, 2263–2268 (2013).
12. Yang, X.Y. *et al.* F33: A-: B-, IncHI2/ST3, and IncI1/ST71 plasmids drive the dissemination of *fosA3* and *bla*$_{CTX-M-55/-14/-65}$ in *Escherichia coli* from chickens in China. *Front Microbiol*. **5**, 688; doi: 10.3389/fmicb.2014.00688 (2014).
13. Miró, E. *et al.* Spread of plasmids containing the *bla*$_{VIM-1}$ and *bla*$_{CTX-M}$ genes and the *qnr* determinant in *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* isolates. *J Antimicrob Chemother*. **65**, 661–665 (2010).
14. Hansen, L. H., Johannesen, E., Burmolle, M., Sorensen, A. H. & Sorensen, S. J. Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in *Escherichia coli*. *Antimicrob Agents Chemother*. **48**, 3332–3337 (2004).
15. Gilmour, M. W., Thomson, N. R., Sanders, M., Parkhill, J. & Taylor, D. E. The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. *Plasmid*. **52**, 182–202 (2004).
16. Bolan, N. S., Khan, M., Donaldson, J., Adriano, D. & Matthew, C. Distribution and bioavailability of copper in farm effluent. *Sci Total Environ*. **309**, 225–236 (2003).
17. Cang, L., Wang, Y., Zhou, D. & Dong, Y. Heavy metals pollution in poultry and livestock feeds and manures under intensive farming in Jiangsu Province, China. *J Environ Sci* (*China*). **16**, 371–374 (2003).
18. Wales, A. D. & Davies, R. H. Co-Selection of Resistance to Antibiotics, Biocides and Heavy Metals, and Its Relevance to Foodborne Pathogens. *Antibiotics*. **4**, 567–604 (2015).
19. Hölzel, C. S. *et al.* Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environl Res*. **113**, 21–27 (2012).
20. Summers, A. *et al.* Mercury released from dental "silver" fillings provokes an increase in mercury-and antibiotic-resistant bacteria in oral and intestinal floras of primates. *Antimicrob Agents Chemother*. **37**, 825–834 (1993).
21. Toleman, M. A. & Walsh, T. R. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev*. **35**, 912–935 (2011).

22. Garcia, A. *et al.* Acquisition and diffusion of *bla*CTX-M-9 gene by R478-IncHI2 derivative plasmids. *FEMS Microbiol Lett.* **271,** 71–77 (2007).
23. Dolejska, M., Villa, L., Minoia, M., Guardabassi, L. & Carattoli, A. Complete sequences of IncHI1 plasmids carrying *bla*CTX-M-1 and *qnrS1* in equine *Escherichia coli* provide new insights into plasmid evolution. *J Antimicrob Chemother.* **69,** 2388–2393 (2014).
24. Cain, A. K. & Hall, R. M. Evolution of IncHI2 plasmids via acquisition of transposons carrying antibiotic resistance determinants. *J Antimicrob Chemother.* **67,** 1121–1127 (2012).
25. Mnif, B. *et al.* Molecular characterization of addiction systems of plasmids encoding extended-spectrum beta-lactamases in *Escherichia coli. J Antimicrob Chemother.* **65,** 1599–1603 (2010).
26. Foster, T. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol Rev.* **47,** 361–409 (1983).
27. Baker-Austin, C., Wright, M. S., Stepanauskas, R. & McArthur, J. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* **14,** 176–182 (2006).
28. Hou, Y. & Taylor, D. E. Incidence of tellurite resistance determinants among plasmids of different incompatibility groups. *Plasmid.* **32,** 306–311 (1994).
29. Johnson, T. J., Wannemuehler, Y. M., Scaccianoce, J. A., Johnson, S. J. & Nolan, L. K. Complete DNA sequence comparative genomics, and prevalence of an IncHI2 plasmid occurring among extraintestinal pathogenic *Escherichia coli* isolates. *Antimicrob Agents Chemother.* **50,** 3929–3933 (2006).
30. Gupta, A., Matsui, K., Lo, J.-F. & Silver, S. Molecular basis for resistance to silver cations in *Salmonella. Nat Med.* **5,** 183–188 (1999).
31. Brown, N. L., Barrett, S. R., Camakaris, J., Lee, B. T. & Rouch, D. A. Molecular genetics and transport analysis of the copper-resistance determinant (pco) from *Escherichia coli* plasmid pRJ1004. *Mol Microbiol.* **17,** 1153–1166 (1995).
32. Deng, H. *et al.* Prevalence of extended-spectrum cephalosporin-resistant *Escherichia coli* in a farrowing farm: ST1121 clone harboring IncHI2 plasmid contributes to the dissemination of *bla*CMY-2. *Front Microbiol.* **6,** 1210; doi:10.3389/fmicb.2015.01210 (2015).
33. Randall, C. P., Gupta, A., Jackson, N., Busse, D. & O'Neill, A. J. Silver resistance in Gram-negative bacteria: a dissection of endogenous and exogenous mechanisms. *J Antimicrob Chemother.* **70,** 1037–1046 (2015).
34. Switt, A. M. *et al.* Identification and characterization of novel *Salmonella* mobile elements involved in the dissemination of genes linked to virulence and transmission. *PloS one.* **7,** e41247–e41247 (2012).
35. Rogers, M., Ekaterinaki, N., Nimmo, E. & Sherratt, D. Analysis of Tn7 transposition. *Mol Gen Genet.* **205,** 550–556 (1986).
36. Mijnendonckx, K., Leys, N., Mahillon, J., Silver, S. & Van Houdt, R. Antimicrobial silver: uses, toxicity and potential for resistance. *Biometals.* **26,** 609–621 (2013).
37. Maillard, J.-Y. & Hartemann, P. Silver as an antimicrobial: facts and gaps in knowledge. *Crit Rev Microbiol.* **39,** 373–383 (2013).
38. Medardus, J. J. *et al.* In-feed use of heavy metal micronutrients in US swine production systems and its role in persistence of multidrug-resistant *salmonellae. salmonellae Appl Environ Microbiol.* **80,** 2317–2325 (2014).
39. Yang, Q. E. *et al.* IncF plasmid diversity in multi-drug resistant *Escherichia coli* strains from animals in China. *Front Microbiol.* **6,** 964; doi: 10.3389/fmicb.2015.00964 (2015).
40. Carattoli, A. *et al.* Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods.* **63,** 219–228 (2005).
41. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. CLSI document M100-S25. (Clinical and Laboratory Standards Institute, 2015).
42. CLSI. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacterial Isolated from Animals. Approved Standard-Fourth Edition and Supplement. CLSI documents VET 01-A4 and VET01-S2. (Clinical and Laboratory Standards Institute, 2013).
43. Deng, Y. *et al.* Dissemination of IncFII plasmids carrying *rmtB* and *qepA* in *Escherichia coli* from pigs, farm workers and the environment. *Clin Microbiol Infect* **17,** 1740–1745 (2011).
44. Chen, S. *et al.* Characterization of multiple-antimicrobial-resistant *salmonella serovars* isolated from retail meats. *Appl Environ Microbiol.* **70,** 1–7 (2004).
45. Barton, B. M., Harding, G. P. & Zuccarelli, A. J. A general method for detecting and sizing large plasmids. *Anal Biochem.* **226,** 235–240 (1995).

Acknowledgements

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

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Fang, L. *et al.* Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from *E. coli* isolates of food-producing animals. *Sci. Rep.* **6**, 25312; doi: 10.1038/srep25312 (2016).