Transmission of plasmid-borne and chromosomal *bla*$_{\text{CTX-M-64}}$ among *Escherichia coli* and *Salmonella* isolates from food producing animals *via* IS*Ecp1*-mediated transposition

Qiu-Yun Zhao[1†], Pin-Xian Chen[1†], Ling-Yang[1], Run-Mao Cai[1], Jia-Hang Zhu[1], Liang-Xing Fang[1,2], Mark A. Webber[3,4] and Hong-Xia Jiang[1,2]

[1]National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, College of Veterinary Medicine; [2]Guangdong Key Laboratory for Veterinary Drug Development and Safety evaluation, College of Veterinary Medicine, South China Agricultural University, Guangzhou, 510642, China; [3]Quadram Institute Bioscience, Norwich Research Park, Norwich, Norfolk, NR4 7UA, UK; [4]Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, Norfolk, NR4 7TJ, UK

[†]These authors contributed equally to this work.

For correspondence:

Hong-Xia Jiang, Ph.D.

National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, College of Veterinary Medicine, South China Agricultural University (SCAU), 483 Wushan Road, Guangzhou, 510642, China

Tel.: +86-020-85284896

Email address: hxjiang@scau.edu.cn

Sir,

Multiple CTX-M variants have been found in the same bacterial host and this co-occurrence within the same cell could favor the formation of CTX-M hybrid enzymes.[1] So far hybrid CTX-M ESBL types have been discovered that include CTX-M-64,[1] M-123,[2] M-132 and M-137,[3,4] and the resulting hybrids have demonstrated higher catalytic activities than their parent enzymes.[5] The CTX-M-1 and M-9 group members were most often found together in *E. coli* from food animals in China suggesting that *E. coli* is the likely host for the generation of novel chimeric alleles. Recently we detected hybrid CTX-M-64 among *Salmonella* from food producing animals, so we questioned whether the occurrence of $bla_{\text{CTX-M-64}}$ in *Salmonella* isolated from food producing animals origin were linked with CTX-M-64 producing *E. coli*. The detection of $bla_{\text{CTX-M-64}}$ in bacterial isolates from both humans and animals in China raises the possibility that a shared environment may be a significant source of co-transmission between animals and humans.[6,7] A first step in determining the transmission mechanisms of hybrid CTX-M enzymes is to identify the genetic contexts of the $bla_{\text{CTX-M-64}}$ genes.

In this study, a total of 435 rectal swab samples from food animals in Guangdong (one chicken farm and two duck farms) and Shandong provinces (one chicken farm) in China in 2016 were collected, of which 276 were obtained from chickens, and 159 from ducks. 329 *E. coli* were obtained, 137 from chickens (41.64%) and 192 from ducks (58.36%), and 60 *Salmonella* were obtained, 39 from chickens (65%) and 21 from ducks (35%). All isolates were screened for the $bla_{\text{CTX-M-64}}$ gene using PCR and sequencing and typed using pulse-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) as previously described.[8] All *Salmonella* isolates were

serotyped using hyperimmune sera by the slide agglutination (S and A Reagents Laboratory, Bangkok, Thailand).

A total of twelve CTX-M-64-positive isolates; three *E. coli*, and nine *Salmonella* were detected. Susceptibility testing by agar dilution of 21 antimicrobial agents showed that all isolates were 100% resistant to ampicillin-cefotaxime-ceftiofur-ceftriaxone-ceftazidime-florfenicol and 75% were resistant to ciprofloxacin (Table 1). The typing data indicated that the 3 *E. coli* isolates were grouped into two PFGE clusters and each cluster had the same novel ST (Figure S1A). The 9 *Salmonella* isolates were grouped into three PFGE clusters designated A, B and C (Figure S1B) and two STs. The STs correlated with specific serovars; ST17 with *S.* Indiana (n=7) and ST19 with *S.* Typhimurium (n=2). Thus, both horizontal transmission and clonal dissemination were responsible for the distribution of the $bla_{\text{CTX-M-64}}$ gene. One additional *S.* Enteritidis isolated that harbored CTX-M-64 was also recently identified from a patient.[9] This indicated that dissemination of $bla_{\text{CTX-M-64}}$ to *Salmonella* strains from different hosts had occurred.

To test the transferability of the $bla_{\text{CTX-M-64}}$ gene, broth mating was performed with a plasmid-free *E. coli* C600 as recipient. Transconjugants were selected on MacConkey agar (Land Bridge, Beijing, China) supplemented with 1 mg/L cefotaxime and 2 mg/L streptomycin. PBRT, S1 nuclease digestion PFGE (S1-PFGE) and Southern hybridization with specific probes confirmed that plasmids were successfully transferred from the 3 *E. coli* isolates and $bla_{\text{CTX-M-64}}$ was located on 65 kb IncI2 plasmids (Figure S2a) for all 3 transconjugants (C-SF1, C-SF4, C-WG20). The plasmid

pWG20 was completely sequenced and the obtained contigs containing *bla*$_{\text{CTX-M-64}}$ indicated that a 3,080-bp IS*Ecp1*-mediated transposition (IS*Ecp1*-*bla*$_{\text{CTX-M-64}}$-*orf477*-A/C) event had occurred and this cassette was 100% identical to a region of pCTXM64_C0967 (Acc. No. KP091735) (Figure 1, Type I).

For the 9 *bla*$_{\text{CTX-M-64}}$-producing *Salmonella* isolates, no transconjugants were obtained, an alternative *E. coli* (DH5α) was used as a recipient for transformation experiments and transformants were selected on Luria-Bertani agar supplemented with 1 mg/L cefotaxime. Only one plasmid was successfully transferred by transformation from DNA from one strain (YC33). Interestingly, two different colony characteristics (a small colony type (T-YC33-1) and a large colony type (T-YC33-2)) were observed for the transformants. The MICs of T-YC33-2 were increased 8- to 16-fold for CTX, CXT and CTR compared with T-YC33-1 (Table S3). PBRT, S1-PFGE and Southern hybridization with *bla*$_{\text{CTX-M-64}}$ and HI2 probes indicated that the *bla*$_{\text{CTX-M-64}}$ gene was present on a 190 kb IncHI2 plasmid (pYC33) in YC33. Unexpectedly, it was located on the chromosome in T-YC33-1 and on a much smaller (less than 20 kb) plasmid (pYC33-2) in T-YC33-2 (Figure S2b and S2c). Significantly, the 2,968-bp region (IS*Ecp1*-*bla*$_{\text{CTX-M-64}}$-*orf477*) was observed in the plasmids pYC33 and pYC33-2 and the chromosome of T-YC33-1 yet was absent from the pYC33-1 plasmids.

Additionally, the pYC33 plasmid carrying *bla*$_{\text{CTX-M-64}}$ was observed partly integrated into the chromosome of T-YC33-1 by PCR mapping (see Table S2 for primers) (Figure S4). This was similar with previous studies where an IncY and

IncA/C2 fusion plasmid harboring *bla*$_{\text{CTX-M-15}}$ was partially integrated into the chromosome of *S. enterica* serotype Concord[10]. In strain T-YC33-2, the 2,968-bp IS*Ecp1*-mediated transposition (IS*Ecp1*-*bla*$_{\text{CTX-M-64}}$-*orf477*) from pYC33 plasmid was integrated into an endogenous ColE-like plasmid (pYC33-1, 3,462-bp) under cefoxitin selective pressure, generating a novel ColE-like plasmid (pYC33-2, 6,435-bp) carrying *bla*$_{\text{CTX-M-64}}$ (Figure 1, Type III; Figure S3).

No transformants were obtained for the remaining 8 *Salmonella* strains despite repeated attempts. The chromosomal location of *bla*$_{\text{CTX-M-64}}$ in these isolates was confirmed by hybridization (Figure S5). In WY36, the 2,968 bp region (IS*Ecp1*-*bla*$_{\text{CTX-M-64}}$-*orf*477) was inserted into the *SgrR* gene (Figure S6) and was similar to the region in YC33 (Figure 1, type IV). However, in another chromosomal location in *Salmonella* isolate SG2, the IS*Ecp1* was truncated by an IS*5* gene and the downstream region of *bla*$_{\text{CTX-M-64}}$ was similar with that of pWG20 in the *E. coli* isolate (Figure 1, Type V). IS*Ecp1*-mediated transposition seems to be responsible for the integration of *bla*$_{\text{CTX-M}}$ gene from plasmid to chromosomal location.[11-14] The high degree of genetic similarity between the *bla*$_{\text{CTX-M-64}}$ plasmids and chromosomes and the frequent reports of *bla*$_{\text{CTX-M-64}}$ in *E. coli* (Figure 1), suggest a common mechanism of IS*Ecp1*-mediated transposition in these strains.[6,7,15] As the IS*Ecp1*-mediated transposition allows horizontal transfer, these elements are likely to further disseminate among *Salmonella* and other bacterial species.

In conclusion, to the best of our knowledge, this is the first report of

chromosomally-encoded CTX-M-64 in *Salmonella* from food animals. These findings indicate that IS*Ecp1*-mediated transposition is likely to be responsible for the spread of *bla*$_{\text{CTX-M-64}}$ between different plasmids and chromosomes in *Enterobacteriaceae* especially *E. coli* and *Salmonella*, resulting in the acceleration of *bla*$_{\text{CTX-M-64}}$ spread. It is imperative that more attention should be paid to the transmission of the *bla*$_{\text{CTX-M-64}}$ gene alone in regional food chain.

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Transparency declarations

None to declare.

Supplementary data

Table S1 to S3, Figure S1 to Figure S5 is available as Supplementary data at JAC Online.

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Table 1. Characteristics of *bla*$_{\text{CTX-M-64}}$ positive *Escherichia coli* and *Salmonella* isolates from chickens and ducks throughout Shandong and Guangdong provinces in 2016

Strain	Species	Serotype	Source	*bla*$_{\text{CTX-M-64}}$ location	Plasmid size (kb)	Replicon type	MLST	Resistance phenotype
SF1[a]	*Escherichia coli*	N/D	Chicken	Plasmid	~360	IncI2	N/T	AMP/CTX/CTF/CTR/CTZ/CHL/FLF/GEN/APR/TET/CIP/DAN/CS/FOM/STR
SF4[a]	*Escherichia coli*	N/D	Chicken	Plasmid	~360	IncI2	N/T	AMP/CTX/CTF/CTR/CTZ/CHL/FLF/GEN/APR/TET/CIP/DAN/CS/FOM/STR
WG20	*Escherichia coli*	N/D	Duck	Plasmid	~360	IncI2	N/T	AMP/CTX/CTF/CTR/CTZ/CHL/FLF/GEN/AMI/APR/TET/DOX/CIP/DAN/CS/STR
YC33	*Salmonella enterica*	Typhimurium	Duck	Plasmid	~190	IncHI2	ST19	AMP/CTX/CTF/CTR/CTZ/GEN/TET/DOX
SG2	*Salmonella enterica*	Typhimurium	Chicken	Chromosome	N/A-	N/A	ST19	AMP/CTX/CTF/CTR/CTZ/CHL/FLF/TET
M77	*Salmonella enterica*	Indiana	Chicken	Chromosome	N/A	N/A	ST17	AMP/CTX/CTF/CTR/CTZ/FLF/TGC/CIP/DAN/FOM
WY6	*Salmonella enterica*	Indiana	Duck	Chromosome	N/A	N/A	ST17	AMP/CTX/CTF/CTR/CTZ/FLF/TGC/CIP/DAN/FOM
WY18	*Salmonella enterica*	Indiana	Duck	Chromosome	N/A	N/A	ST17	AMP/CTX/CTF/CTR/CTZ/FLF/TGC/CIP/DAN/FOM
WY23	*Salmonella enterica*	Indiana	Duck	Chromosome	N/A	N/A	ST17	AMP/CTX/CTF/CTR/CTZ/FLF/TGC/CIP/DAN/FOM
WY29	*Salmonella enterica*	Indiana	Duck	Chromosome	N/A	N/A	ST17	AMP/CTX/CTF/CTR/CTZ/FLF/TGC/CIP/DAN/FOM
WY35	*Salmonella enterica*	Indiana	Duck	Chromosome	N/A	N/A	ST17	AMP/CTX/CTF/CTR/CTZ/FLF/TGC/CIP/DAN/FOM
WY36	*Salmonella enterica*	Indiana	Duck	Chromosome	N/A	N/A	ST17	AMP/CTX/CTF/CTR/CTZ/FLF/TGC/CIP/DAN/FOM

N/D: not determined; N/T: not typeable; N/A: not applicable;

AMP, ampicillin; CTX, cefotaxime; CTF, ceftiofur; CTR, ceftriaxone; CTZ, ceftazidime; CHL, chloramphenicol; FLF, florfenicol; GEN, gentamycin; APR, apramycin; TGC, tigecycline; AMI, amikacin; CIP, ciprofloxacin; DAN, danofloxacin; TET, tetracycline; DOX, deoxytetracycline; CS, colistin; FOM, fosfomycin; STR, streptomycin.

Common resistance phenotype was AMP, CTX, CTF, CTR, CTZ, FLF, TGC, CIP, DAN, and FOM.

[a] *bla*$_{\text{CTX-M-64}}$ and *bla*$_{\text{CTX-M-65}}$ co-exist in the same *Escherichia coli* strain.

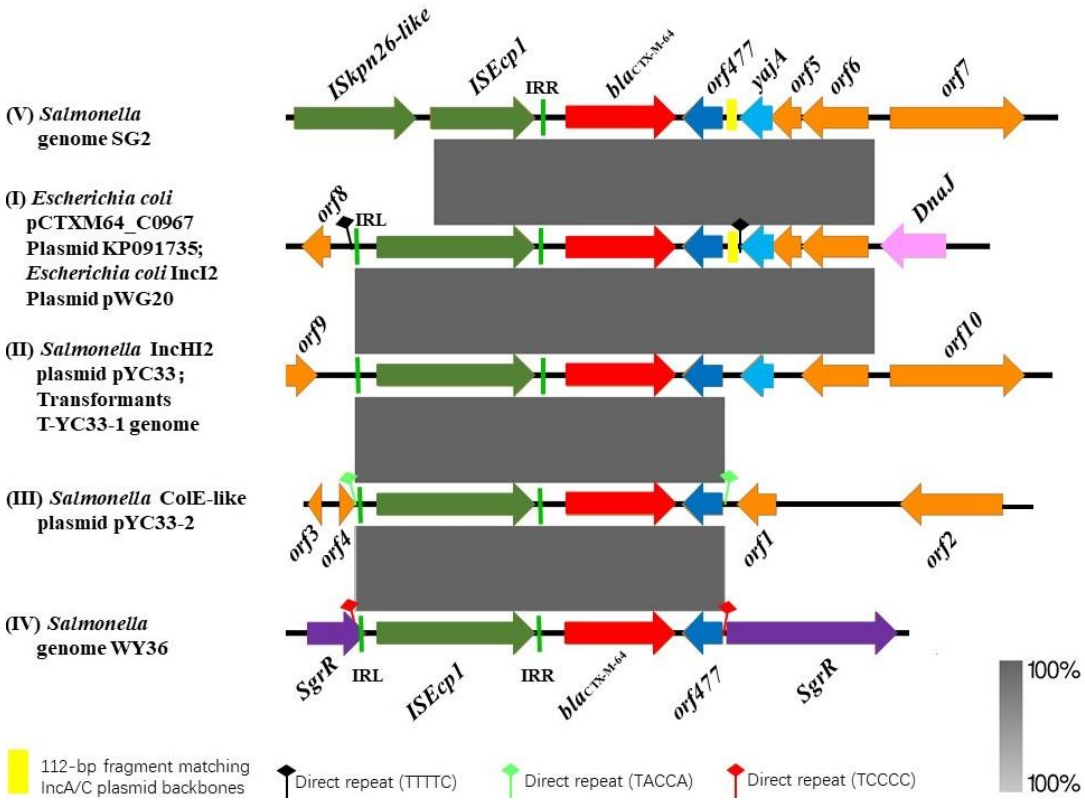

Figure 1. Genomic and molecular analyses for *bla*$_{CTX-M-64}$-positive plasmids.

(A) Genomic environment of the *bla*$_{CTX-M-64}$ gene in *Escherichia coli* isolates and *Salmonella* isolates. (I) Genetic environment of *bla*$_{CTX-M-64}$ gene of pCTXM64_C0967 (KP091735) and *E. coli* isolates pWG20. (II) Genetic environment of *bla*$_{CTX-M-64}$ gene of plasmid pYC33 and T-YC33-1. (III) Genetic environment of *bla*$_{CTX-M-64}$ gene of ColE-like plasmid pYC33-2. (IV) Genetic environment of *bla*$_{CTX-M-64}$ gene in the chromosome of *Salmonella* isolate WY36. (V) Genetic environment of *bla*$_{CTX-M-64}$ gene in the chromosome of Salmonella isolate SG2.

Table S1 Distribution of CTX-M subgroups and alleles amongst *Escherichia coli* and *Salmonella* isolates

Species	ESBL(s) gene			No. of isolates
	bla$_{\text{CTX-M-1}}$ Group	*bla*$_{\text{CTX-M-9}}$ Group	hybrid gene	
Escherichia coli	*bla*$_{\text{CTX-M-15}}$			6
	bla$_{\text{CTX-M-109}}$			1
	bla$_{\text{CTX-M-79}}$			14
	bla$_{\text{CTX-M-55}}$			16
		bla$_{\text{CTX-M-14}}$		21
		bla$_{\text{CTX-M-24}}$		3
		bla$_{\text{CTX-M-27}}$		26
		bla$_{\text{CTX-M-65}}$		39
			bla$_{\text{CTX-M-64}}$	3
			bla$_{\text{CTX-M-123}}$	2
Salmonella enterica		*bla*$_{\text{CTX-M-27}}$		3
		bla$_{\text{CTX-M-65}}$		2
			bla$_{\text{CTX-M-64}}$	9

Two *bla*$_{\text{CTX-M}}$ genes (CTX-M-1 group and CTX-M-9 group) coexist in the same *Escherichia coli*: *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-14}}$ (2 strains), *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$ (3 strains), *bla*$_{\text{CTX-M-65}}$ and *bla*$_{\text{CTX-M-64}}$ (2 strain).

Table S2 Selected primers used in this study.

Primer	Nucleotide sequence (5′→3′)	Target DNA sequence	Reference/Source
R-pYC33-2F	CTGGTTCTCCTTCCGCTG	*orf477*	This study
R-pYC33-2R	ACACTCCCTTGTACGGATAG	IS*Ecp1*	
pYC33-1F	GGTGTCATTCCGCTGTTAT	pYC33-2 backbone	This study
pYC33-1R	TAGTCCTGTCGGGTTTCGC		
U-pYC33 -F	CCTGGTTTTTGGGGTTGAT	*ThrG*	This study
U-pYC33 -R	TAGGTTGAGGCTGGGTGAA	*bla*$_{\text{CTX-M-64}}$	
D-pYC33 -F	TGATTCTGGTCACTTACTT	*bla*$_{\text{CTX-M-64}}$	This study
D-pYC33 -R	TCTATGCTTCTCCATTTCT	*Orf10*	
16S rRNA-F	GGCCGCAAGGTTAAAACTCAAATG	*16S rRNA* for quantitative	1
16S rRNA-R	AACCGCTGGCAACAAAGGATAAGG	real-time PCR	
qCTX-M-64-F	CTGGGTTGTGGGGGAT	qCTX-M-64 for	This study
qCTX-M-64-R	GGTTGAGGCTGGGTGA	quantitative real-time PCR	

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Table S3 Characteristics of the YC33 *Salmonella* strain and its transformants.

Strains	MIC (μg/mL)					Gene location of $bla_{CTX\text{-}M\text{-}64}$
	CTF	CTX	CTZ	CXT	CTR	
YC33	>512	512	128	4	256	IncHI2 plasmid pYC33
T-YC33-1	>512	16	8	4	16	chromosome
T-YC33-2	>512	256	64	4	256	ColE-like plasmid pYC33-1

Note: Transformants were challenged for the transformation by using recipients DH5α.

Abbreviations: CTX, cefotaxime; CXT, cefoxitin; CTZ, ceftazidime; CTR, ceftriaxone; MIC, minimal inhibitory concentration.

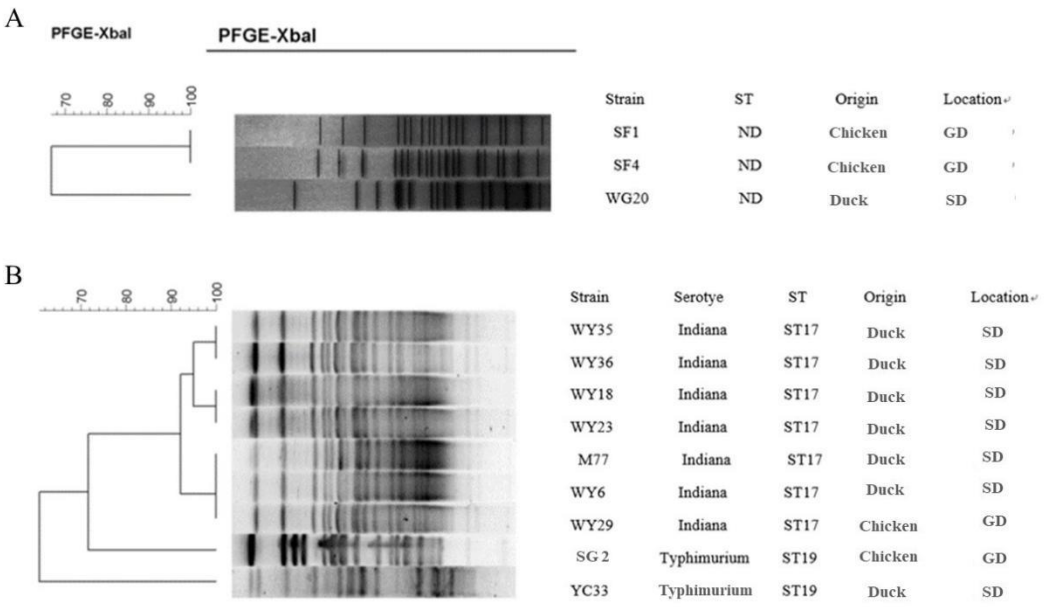

Figure S1 PFGE dendrogram showing the CTX-M-64-positive *Escherichia coli* and *Salmonella* isolates.

(A) PFGE dendrogram showing the CTX-M-64-positive *Escherichia coli* isolates. (B) PFGE dendrogram showing the CTX-M-64-positive *Salmonella* isolates. GD, Guangdong province; SD, Shandong province. ND: not determined.

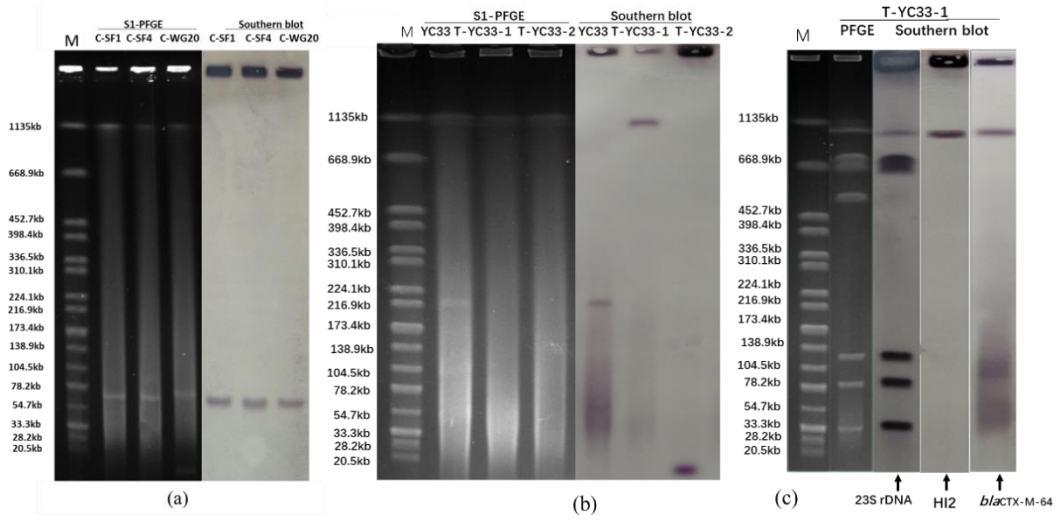

Figure S2. Southern hybridization of *bla*$_{\text{CTX-M-64}}$-positive plasmids and chromosome.

(a) The S1-PFGE electrophoretic profiles of the plasmids in transconjugants and hybridization with the *bla*$_{\text{CTX-M-64}}$-specific probes; (b) The S1-PFGE electrophoretic profiles of the plasmids in transformants and hybridization with the *bla*$_{\text{CTX-M-64}}$-specific probes; (c) The *I-Ceu*1-PFGE electrophoretic profiles of transformant T-YC33-1 and southern blot hybridization with 23S rDNA probes and *bla*$_{\text{CTX-M-64}}$ probes. M: Marker.

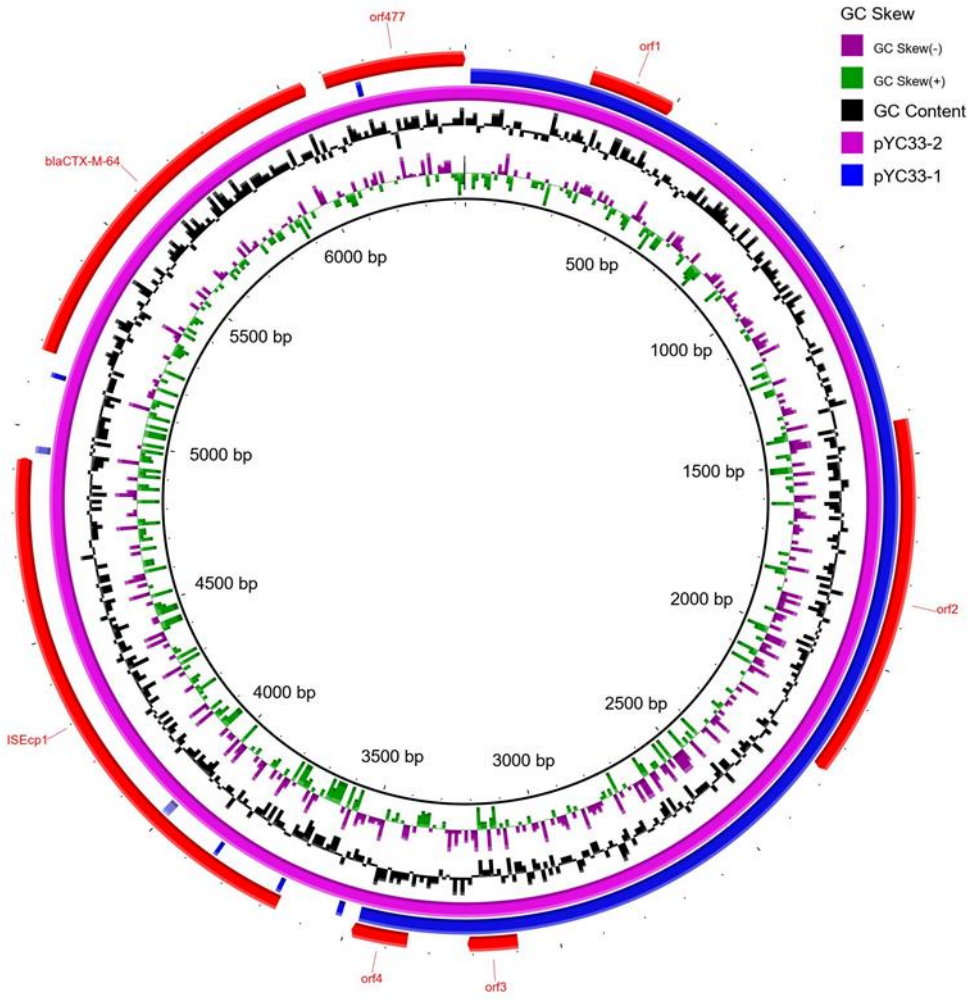

Figure S3 The size of pYC33-2 plasmid and the comparison of pYC33-2 and pYC33-1.

Comparison of ColE-like pYC33-2 and ColE-like pYC33-2. The comparison is a pairwise BLASTn alignment performed using BRIG.

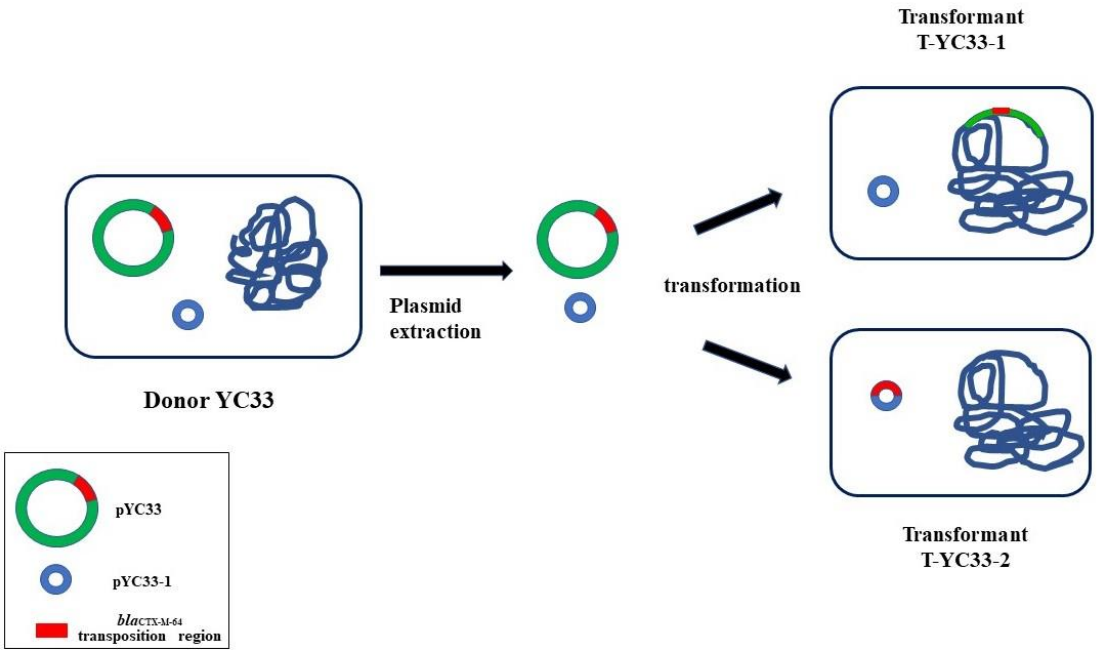

Figure S4. Schematic representation of the transfer of the plasmid-borne *bla*$_{CTX-M-64}$ gene into transformants.

On the left side is the donor strain. On the right side is the transformants involved in transformation.

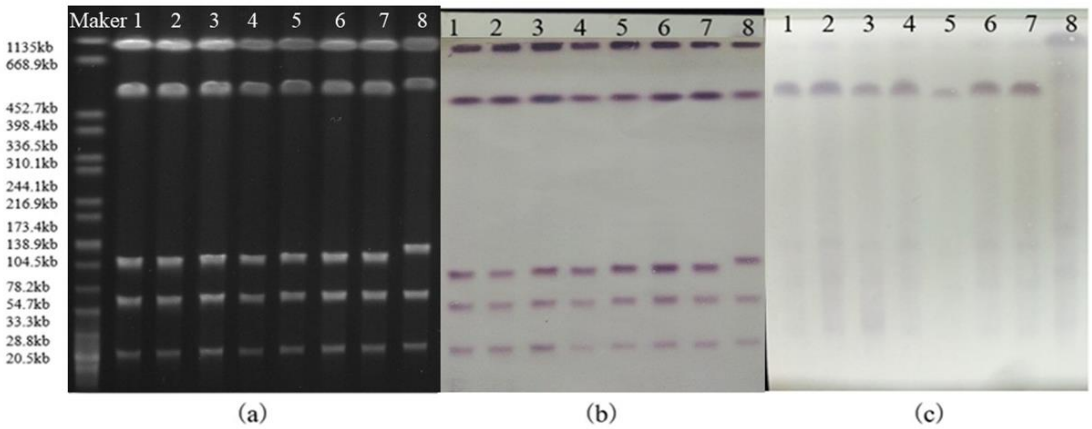

Figure S5 Chromosomal location of *bla*$_{\text{CTX-M-64}}$ in the *Salmonella* isolates using *I-Ceu*1-PFGE and Southern blot hybridization

(a) The *I-Ceu*1-PFGE electrophoretic profiles of *Salmonella* chromosome DNA; (b) Southern blot hybridization with 23S rDNA probes; (c) Southern blot hybridization with *bla*$_{\text{CTX-M-64}}$ probes. M: PFGE Marker; Lanes 1-8, strains M77, WY6, WY18, WY23, WY29, WY35, WY36 and SG2, respectively.

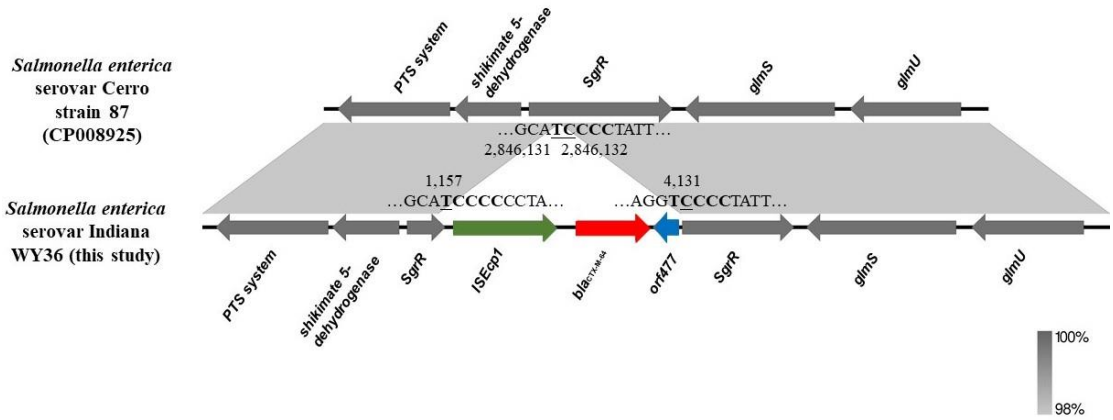

Figure S6. Schematic representation of insertion of transposition units harboring *bla*$_{\text{CTX-M-64}}$ in the chromosome from *Salmonella*.

The numbers above the color arrows correspond to nucleotide numbers in the annotation of the genetic context of *bla*$_{\text{CTX-M-64}}$ in WY36. The numbers underneath the grey arrows correspond to nucleotide numbers in the annotation of the reference *Salmonella enterica* CERRO strain 87 (CP008925). The surrounding nucleotide sequences at the insertion points are shown. The 2968-bp transposition unit sequence is indicated in color arrows, and the duplicated sequences generated during the transposition events are highlighted in boldface (TCCCC).