Clonal spread of \textit{mcr-1} in PMQR-carrying ST34 \textit{Salmonella} isolates from animals in China

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Since initial identification in China, the widespread geographical occurrence of plasmid-mediated colistin resistance gene \textit{mcr-1} in \textit{Enterobacteriaceae} has been of great concern. In this study, a total of 22 \textit{Salmonella enterica} were resistant to colistin, while only five isolates which belonged to ST34 \textit{Salmonella enterica} serovar Typhimurium (\textit{S. Typhimurium}) were \textit{mcr-1} positive. Four of them shared nearly identical PFGE type, although they were from different host species and diverse geographical locations. All the \textit{mcr-1}-positive \textit{S. Typhimurium} exhibited multi-resistant phenotypes including ampicillin, streptomycin, gentamicin, florfenicol, nalidixic acid, tetracycline, trimethoprim-sulfamethox, in addition to colistin. The \textit{qoxAB} and \textit{aac(6\prime)-Ib-cr} genes were present alone or in combination in four (80.0%) and five (100%) isolates, respectively. The \textit{mcr-1} gene was located on a transferable IncI2 plasmid in the four genetically related strains. In the other one strain, \textit{mcr-1} was located on an approximately 190 kb IncHI2 plasmid. In conclusion, we report five \textit{mcr-1}-positive \textit{S. Typhimurium}/ST34 isolates. Both clonal expansion and horizontal transmission of IncI2-type plasmids were involved in the spread of the \textit{mcr-1} gene in \textit{Salmonella enterica} from food-producing animals in China. There is a great need to monitor the potential dissemination of the \textit{mcr-1} gene.

Salmonellosis is one of important global public health zoonoses, causing life-threatening infections. Each year, there are an estimated 1.0 million \textit{Salmonella} infections in the United States\textsuperscript{1}. By contrast, 30 millions of infections every year in China, approximately 75% of the food-borne diseases, are attributed to this bacterium\textsuperscript{2}. Unpublished data from the China CDC surveillance system indicated that the carriage rate of human salmonellosis is 549 per 100,000 people in 2013, which is higher than that in the USA in 2012 (16.4 per 100,000)\textsuperscript{3}. \textit{Salmonella enterica}, especially non-typhoidal \textit{Salmonella} (NTS), is a leading cause of food–borne disease of humans and livestock worldwide\textsuperscript{4,5}. Various animal species, such as poultry, pigs, cattle, and reptiles, are reservoirs for NTS. Human NTS infections are frequently due to the consumption of contaminated cooked or raw meat, milk, eggs, seafood, and other fresh products derived from animals\textsuperscript{6}.

In recent year, emerging fluoroquinolone resistance prevalence has been identified in several \textit{Salmonella} serovars and the resistance rate to fluoroquinolone has increased dramatically both in clinical and food-borne \textit{Salmonella} isolates around the world\textsuperscript{6}. Resistance toward quinolone and fluoroquinolone antimicrobials is mainly attributed to mutations of quinolone resistance-determining regions (QRDRs) and plasmid-mediated quinolone resistance (PMQR) mechanism including Qnr peptides (QnrA, QnrB, QnrS, QnrD and QnrC), AAC(6\prime)-Ib-cr and the efflux pumps QepA and OqxAB\textsuperscript{7}. In our previous study, we have characterized a high prevalence of \textit{qoxAB} (31.7\%) in \textit{Salmonella enterica} serotype Typhimurium (\textit{S. Typhimurium}) isolated from food-producing animals in China. The \textit{qoxAB} gene was present alone or in combination with other PMQR genes such as \textit{aac(6\prime)-Ib-cr} and \textit{qnrS1} genes. Interestingly, the \textit{S. Typhimurium} isolates carrying \textit{qoxAB} were clonally related as determined by PFGE and also defined as ST34 by MLST type\textsuperscript{8}. A high prevalence of \textit{qoxAB}-positive \textit{S. Typhimurium}/ST34 was also detected in human clinical and food samples in Hong Kong in the same period\textsuperscript{9}. It aroused a possibility that the \textit{qoxAB}-positive \textit{S. Typhimurium}/ST34 transmitted from food animals to humans via food chain.

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More recently, *Salmonella* species have been isolated that carried PMQR, can occur as a multiple drug resistance phenotype, which has caused international concern because it brought an even greater challenge for clinical treatment9,10.

Colistin (polymyxin E) is a cationic, multi-component, lipopeptide antibacterial agent discovered in the 1940s with significant activity against Gram-negative bacteria. Colistin has been used both in human and veterinary medicine for more than 50 years, although their parenteral usage in humans has been limited because of concerns about nephrotoxicity and neurotoxicity11. However, with a global increase in Gram-negative bacteria that are multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR), colistin has been re-introduced as a last-resort drug for infections with such bacteria, which are frequently the cause of healthcare-associated infections12,13. In veterinary medicine, colistin is more widely used, mainly to treat Gram-negative infections of the intestinal tract14. In addition, colistin is used as a growth promoter in some countries due to its great growth performance in pig and poultry production14,15.

Resistance to colistin in Gram-negative bacteria has been characterized by chromosomal mutations and was generally thought non-transferable by mobile genetic elements11. Specific regions mutations like *pmrAB* and *phoPQ*, which were related to structural changes (ParR-ParS two-component system) of the LPS at both the cytosol and periplasmic site of the cell membrane, could decrease colistin activity in *Klebsiella*, *E. coli* and *Salmonella enterica*14.

Recently, a plasmid-mediated colistin resistance gene (*mcr-1*) was firstly reported in food animals, food and humans in China15. The *mcr-1* gene was proved subsequently to be disseminated worldwide, mainly found in *E. coli*, *K. pneumonia*, *Enterobacter aerogenes*, and *Enterobacter cloacae* in many regions (Fig. 1), mainly in Guangdong15,17,18, Shanghai15, Zhejiang, Hubei, Jiangsu19, Sichuan20, Shandong, Anhui21, Chongqing22, Hong Kong23, and Taiwan24, whereas data on the transmission of *mcr-1*-mediated colistin resistance in *Salmonella spp.* are lacking. Although, during the preparation of this study, the prevalence of *mcr-1* among ESBL-positive *Salmonella* *spp.* isolates was investigated, but the presence of this gene in particular successful resistant clone has not been demonstrated25. In this study, we did a retrospective study to examine the emergence of the *mcr-1* gene in *Salmonella enterica* isolates from food-producing animals during 2007 to 2015.

**Results**

**Antimicrobial susceptibility and detection of resistance genes.** These isolates showed the minimal inhibitory concentrations (MIC) for colistin of 0.25 to 16 mg/L. A total of 22 *Salmonella* isolates were resistant to colistin with MIC $\geq 8$ mg/L. The *mcr-1* gene was detected in only five colistin-resistant *S. Typhimurium* isolates with diverse origins (Table 1). Susceptibility testing showed that all the *mcr-1*-positive isolates in this study were resistant to nalidixic acid, olaquindox, ampicillin, streptomycin, gentamicin, florfenicol, tetracycline and trimethoprim-sulfamethox. All the five *mcr-1*-containing strains carried *aac(6’)-Ib-cr*, and four of them also carried *qepAB* and *floR* (Table 1). Interestingly, all strains showed increased MIC values and exhibited intermediate resistance phenotype (strain GDS 79, GDS82, and GDS141) or resistance phenotype (strain S01) but except strain GDS78 retained sensitivity to ciprofloxacin according to the cutoff of CLSI, although most of them harboured one or more PMQR genes (Table 1).

**Transfer of *mcr-1* gene.** Four transconjugants were successfully obtained from the five *mcr-1*-positive isolates. Conjugation and transformation tests were not successful for strain S01 despite repeated attempts. All
transconjugants showed 32-fold increase in the MICs of colistin, in comparison with the recipient *E. coli* C600 (0.125 mg/L). However, other antibiotic-resistant phenotypes could not be co-transfered with colistin except for strain GDS78. The transconjugant of GDS78 was multidrug-resistant and showed resistance to more than five antibiotics, in addition to colistin (Table 1).

**Table 1. Characteristics of the five *Salmonella enterica* isolates carrying mcr-1.**

| Straina | Colistin MIC | Other resistant profileb | Resistance gene(s)b | Plasmid |
|---|---|---|---|---|
| S01 | 16 | OLA, CIP, NAL, AMP, STR, GEN, FFC, TET, SMZ/TMP | *mcr-1*, oqxAB, *aac(6′)-Ib-cr*, floR | H12 | ~190 |
| GDS78 | 16 | OLA, NAL, AMP, STR, GEN, FFC, TET, SMZ/TMP | *mcr-1*, *aac(6′)-Ib-cr* | H12, I2, FIB, FII | ~70 |
| GDS79 | 16 | OLA, NAL, AMP, STR, GEN, FFC, TET, SMZ/TMP | *mcr-1*, oqxAB, *aac(6′)-Ib-cr*, floR | H12, I2, FIB, FII | ~70 |
| GDS82 | 16 | OLA, NAL, AMP, STR, GEN, FFC, TET, SMZ/TMP | *mcr-1*, oqxAB, *aac(6′)-Ib-cr*, floR | H12, I2, FIB, FII | ~70 |
| GDS141 | 16 | OLA, NAL, AMP, STR, GEN, FFC, TET, SMZ/TMP | *mcr-1*, oqxAB, *aac(6′)-Ib-cr*, floR | H12, I2, FIB, FII | ~70 |

*S. enterica* isolates from which the *mcr-1* gene can be transferred to the recipient by conjugation are underlined. AMP, cefotaxime; STR, streptomycin; GEN, gentamicin; FFC, florfenicol; OLA, olaquindox; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; SMZ/TMP, trimethoprim/sulfamethoxazole. The antimicrobial susceptibility results were interpreted according to breakpoint of CLSI (M100-S25), except that florfenicol (≥32 μg/mL) was interpreted according to breakpoint of veterinary CLSI (VET01-A4/VET01-S3). All isolates were susceptible to amikacin, cefotaxime, cefoxitin, fosfomycin, and meropenem. Resistance phenotypes, genes and plasmids transferred to the recipient by conjugation are underlined.

**Figure 2.** (A) Pulsed field gels of *S1* digested genomic DNA and Southern blot in gel hybridization with probe *mcr-1*. (B) ApaLI restriction digestion profiles of *mcr-1*-carrying plasmids of the three transconjugants.
of the mcr-1 gene (Table 2). In the original strain S01, we then confirmed that a 3,679 bp length of ISApl1-mcr-1 fragment was inserted in approximately 8,500 bp downstream of terY2 on pHNSHP45-2 (KU341381), which is in accordance with pMR0516mcr (KX276657) (Fig. 3).

**Molecular typing.** All of the five isolates were successfully typed by pulsed-field gel electrophoresis (PFGE), and two different PFGE clusters designated A and B were obtained (Fig. 4). Cluster A contains four isolates, three of which were isolated from different pig farms in 2008 and 2009, while the remaining one was isolated from duck in 2010. The single strain of cluster B was isolated from chicken in 2007. Multi-locus sequence typing showed that the five mcr-1-positive S. Typhimurium belongs to ST34, though they were classified into two PFGE clusters (Fig. 4).
isolates are sporadic in animals in China. It is possible that the transferable strains belong to *Salmonella* food chain might expedite colistin resistance in the same period. The dissemination of this clone carrying *Salmonella* showed that the plasmid-mediated colistin resistance was not the main way conferred colistin resistance among *Bacterial strains*.

### Materials and Methods

#### Bacterial strains

A total of 276 nonduplicate *Salmonella enterica* isolates (246 from avian and 30 from swine), isolated from faecal swabs of healthy or sick animals at poultry farms, swine farms and two diagnostic laboratories in Guangdong and Shandong province in China among 2007 and 2015, were used in this study (Table 3). Among them 127 were *S. Typhimurium*, 79 were *Salmonella enterica* serotype Enteritidis, two were *Salmonella enterica* serotype Enteritis, six were *Salmonella enterica* serotype Indiana, six were *Salmonella enterica* serotype Meleagridis, two were *Salmonella enterica* serotype Bredeney, one was *Salmonella enterica* serotype Abaetetuba and 59 were non-typeble. The details of these strains have been described in our previous works.

### Discussion

Since initial identification in China, the *mcr-1* gene has been detected in *Enterobacteria* from almost 30 countries on five continents. The *mcr-1* gene was frequently detected in *Enterobacteriaceae*, including *E. coli*, *Enterobacter aerogenes* and *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Shigella somnus*, as well as *Salmonella enterica*. Among them, *Salmonella enterica* has attracted much attention owing to it as an important food-borne pathogen. *Salmonella enterica* harbour a number of serovars, but the *mcr-1* is not restricted to a certain serovar, which has been identified in *Salmonella enterica* serotype Anatum, Derby, 1,4,[5],12:i:2,31 Java, Paratyphi B, Rissen, Schwartzengrund, Typhimurium and Virchow. Moreover, several reports confirmed the *mcr-1*-positive *Salmonella enterica* were multidrug resistance strains, which usually carry other resistance genes including ESBL genes and quinolone resistance genes. More recently, the *mcr-1* gene was found in the multidrug resistant and copper-tolerant *Salmonella enterica* serotype Typhimurium/ST34, which is the predominant ST of *S. Typhimurium* in Guangdong, China. In our previous study, we have characterized a high prevalence of *Mdr* S. *Typhimurium*/ST34 carrying PMQR, which was also frequently detected in human clinical and food samples in Hong Kong in the same period. The dissemination of this clone carrying *mcr-1* from food-producing animals to humans via food chain might expedite colistin resistance in *Salmonella enterica*.

The *mcr-1* gene was so far associated with diverse plasmids belonging to the IncI2, IncHI2, IncP, IncF replicon types such as IncHI2 and IncFII in the four clonal strains. The *mcr-1* gene was only found on IncI2 plasmids in this study. This indicated that other mobile elements were probably involved in the mobilization of the *mcr-1* gene, in addition to plasmids. Indeed, IS*Ap1* was present upstream of the *mcr-1* gene on both *mcr-1*-carrying plasmids in *E. coli* SHP45, while it was absent on all the *mcr-1* positive IncI2 plasmids in this study.

Only five *mcr-1*-positive strains were identified in 276 isolates, indicating that the *mcr-1*-positive *Salmonella enterica* isolates are sporadic in animals in China. It is possible that the transferable *mcr-1* gene here appears in the form lacking the insertion sequence IS*Ap1*. In addition, all the *mcr-1*-positive *Salmonella enterica* were isolated from the disease animals, whereas most of the *mcr-1*-negative colistin resistant strains were from healthy animals. Although the prescription was not recorded during the sampling, existing evidence suggested that exposure to colistin was closely related to its resistance rates. The high rate of colistin resistance and low *mcr-1*-positive rates showed that the plasmid-mediated colistin resistance was not the main way conferred colistin resistance among *Salmonella enterica* isolates. It is of possibility that other colistin-resistant mechanisms or even novel *mcr-1* type exist, which needs to be evaluated in future study.

In conclusion, we reported five *mcr-1*-positive *Salmonella enterica* isolates from animals in China between 2007 and 2015. Clonal spread of PMQR-carrying ST34 *Salmonella enterica* isolates and horizontal transmission of IncI2 plasmids were the main way to disseminate *mcr-1* gene in this study. Colistin should be used more prudently in food-producing animals to prevent *mcr-1* gene spreading between different species.
agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institution. All the mcr-1-positive isolates were detected by other resistance determinants including PMQR genes, rmtB, armA and floR. Susceptibility testing was also assessed in the mcr-1-positive isolates using the following antimicrobial agents: ampicillin (AMP), cefotaxime (CTX), cefoxitin (FOX), meropenem (MEM), streptomycin (STR), amikacin (AMK), gentamicin (GEN), florfenicol (FFC), tetracycline (TET), nalidixic acid (NAL), ciprofloxacin (CIP), olaquindox (OQX), trimethoprim-sulfamethox (SMZ/TMP), and fosfomycin (fos). E. coli ATCC 25922 was used as a quality control strain.

**Screening of the mcr-1 gene and susceptibility testing.** MICs of Colistin were determined by the agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institution. Colistin-resistant isolates were screened for the presence of mcr-1 by PCR with the primers as described by Liu et al. All the mcr-1-positive isolates were detected by other resistance determinants including PMQR genes, rmtB, armA and floR. Susceptibility testing was also assessed in the mcr-1-positive isolates using the following antimicrobial agents: ampicillin (AMP), cefotaxime (CTX), cefoxitin (FOX), meropenem (MEM), streptomycin (STR), amikacin (AMK), gentamicin (GEN), florfenicol (FFC), tetracycline (TET), nalidixic acid (NAL), ciprofloxacin (CIP), olaquindox (OQX), trimethoprim-sulfamethox (SMZ/TMP), and fosfomycin (FOS). E. coli ATCC 25922 was used as a quality control strain.

**Conjugation and transformation analysis.** E. coli C600 was used as the recipient for the conjugation experiment of MCR-producing Salmonella isolates. The transconjugants were selected on MacConkey agar containing colistin (2 mg/L) and streptomycin (2,000 mg/L), and finally confirmed by PCR and ERIC-PCR. Plasmids that are not transferable by conjugation were studied by transformation assay. Plasmid DNA was extracted using a QIAGEN Prep Plasmid Midi Kit. Purified plasmids were used in electroporation experiments with E. coli DH5α following the manufacturer’s instructions. Transformants were incubated at 37°C for 1 h and were then selected on LB agar containing 2 mg/L colistin.

**Plasmid Characterization.** Incompatibility (Inc) groups were assigned by PBRT. To analyze the location of the mcr-1 gene, S1 nuclease-PFGE and Southern blot analysis were performed. Briefly, whole-cell DNA of the donor strains and the transconjugants harbouring mcr-1 were extracted and embedded in agarose gel plugs. Subsequently, the agarose gel plugs were treated with S1 nuclease (TaKaRa, Dalian, China) and the DNA fragments were separated by PFGE. Southern blot hybridization was then performed with DNA probes specific for the mcr-1 gene, which was non-radioactively labeled with a DIG High Prime DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany). Transconjugants containing one plasmid were extracted and analyzed by RFLP using ApaI (TaKaRa, Dalian, China) digestion. The genetic context surrounding the mcr-1 gene was investigated by PCR mapping and sequencing according to the plasmids sequences which had been submitted to GenBank. The primers used to determine the regions upstream and downstream of the mcr-1 gene were then selected on LB agar containing 2 mg/L colistin.

**Molecular typing.** Genomic DNA of the mcr-1-positive isolates was analyzed by PFGE following digestion with XbaI. Salmonella enterica serotype Braenderup H9812 standard was used as size marker. Comparison of PFGE patterns was performed by BioNumerics® v6.6 (Applied Maths, Ghent, Belgium) with a cut-off at 90% of the similarity values to indicate identical PFGE types. Multi-locus sequence typing (MLST) was performed by using the primers and protocol specified at the Salmonella enterica MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

**Table 3.** Colistin resistant and mcr-1 positive Salmonella strains from animals in China, 2007–2015. *N.a.* means not available.

| Year | Animals | Strains tested for colistin MIC | Resistant to colistin | Proportion of mcr-1 positive (n) among colistin-resistant Salmonella strains (N) |
|------|---------|-------------------------------|----------------------|--------------------------------------------------------------------------------|
| 2007 | Avian   | 44                            | 2                    | 1/2                                                                            |
| 2008 | Avian   | 53                            | 5                    | 0/5                                                                            |
| 2008 | Swine   | 7                             | 3                    | 2/3                                                                            |
| 2009 | Avian   | 28                            | 1                    | 0/1                                                                            |
| 2009 | Swine   | 3                             | 1                    | 1/1                                                                            |
| 2010 | Avian   | 11                            | 4                    | 1/4                                                                            |
| 2010 | Swine   | 3                             | 0                    | N.a.                                                                          |
| 2012 | Avian   | 42                            | 5                    | 0/5                                                                            |
| 2014 | Avian   | 17                            | 0                    | N.a.                                                                          |
| 2014 | Swine   | 11                            | 0                    | N.a.                                                                          |
| 2015 | Avian   | 51                            | 1                    | 0/1                                                                            |
| 2015 | Swine   | 6                             | 0                    | N.a.                                                                          |

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**Acknowledgements**

This work was supported by the National Natural Science Foundation and the Natural Science Foundation of Guangdong Province, China (grant U1201214), the Program for Changjiang Scholars and Innovative Research Team at the University of the Ministry of Education of China (grant IRT13063), and the Natural Science Foundation of Guangdong Province (grant S2012030006590), and the Basic Research Program of China (2016YFC1200100). Dr. Feng is a recipient of the “Young 1000 Talents” Award.

**Author Contributions**

X.-P.L. performed experiments, analyzed the data and wrote the main manuscript; L.-X.F. and Y.F. edited the manuscript; J.-Q.S., W.H. and J.-T.F. performed experiments; J.X. analyzed the data; X.-P.L. and Y.-H.L. coordinated the whole project; J.S. designed this project, analyzed the data and revised the article.

**Additional Information**

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

**How to cite this article:** Li, X.-P. et al. Clonal spread of mcr-1 in PMQR-carrying ST34 Salmonella isolates from animals in China. *Sci. Rep.* 6, 38511; doi: 10.1038/srep38511 (2016).

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