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SHORT REPORT

Genetic Characterization of Colistin-Resistant *Salmonella enterica* ST34 Co-Harbouring Plasmid-Borne *mcr-1*, $bla_{CTX-M-15}$ and bla_{KPC-2} Recovered from a Paediatric Patient in Shenzhen, China

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Background: Since 2015, plasmid-borne *mcr-1* has been reported in various bacterial strains in the clinical setting globally. However, the transmission mechanisms of this gene in *Salmonella* are not well defined. This study aimed to characterize the genomic features of a *Salmonella enterica* ST34 isolate, which carried a *mcr-1*, mapped to a carbapenemase and extended spectrum β-lactamase encoding gene located on the IncX4 plasmid.

Methods: *Salmonella enterica* was recovered from a diarrheal paediatric patient in Shenzhen, China. Antimicrobial susceptibility testing was performed by using the VITEK 2 system. Drug resistance genes were identified using targeted primers and Sanger sequencing. The transferability and genome location of *mcr-1* was determined by performing conjugation, S1-PFGE and Southern blot hybridization analysis. WGS was performed by Illumina MiSeq sequencing and was assembled using the A5-Miseq pipeline, and gene annotation was performed using RAST 2.0. The database Centre for Genomic Epidemiology's website was used to identify resistance genes and sequence types (STs).

Results: We found that the isolate was extensively drug resistant and belonging to ST34, carrying an IncX4 plasmid with *mcr-1,* bla_{KPC-2} and $bla_{CTX-M-15}$. We also noticed that genes bla_{PAO}, *fosA, catB*, the mutation in *oprD* and *mexT* (MexEF-OprN efflux regulator), and exotoxin-encoding genes (*exoS, exoY* and *exoT*) were associated with resistance and virulence in the genome. In addition, heavy metal resistance genes as *silP and silE* were determined.

Conclusion: This study highlights the potential risk of ST34 of *Salmonella enterica* serotype Typhimurium carrying multiple drug resistance encoding genes in a single IncX4 plasmid.

Keywords: *Salmonella enterica*, MCR-1, KPC-1, CTX-M-15, paediatric patient

Introduction

Acute diarrheal diseases are associated with significant mortality and morbidity.[1] In the year 2018, the World Health Organization (WHO) reported more than 2 billion people globally that suffered from diarrheal disease.[1] *Salmonella* species are becoming a major global public health concern. These species cause a broad range of clinical conditions, the most common is gastroenteritis, followed by bacteraemia and enteric fever.[2] Colistin is commonly used as a last choice of

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drug to treat infections caused by multi-drug resistant (MDR) or extensively drug-resistant (XDR) bacteria.[3] Extended-spectrum beta-lactamase (ESBLs) is prominently encoded by $bla_{\text{CTX-M-15}}$ gene and hydrolyses the penicillin, third-generation cephalosporin. Carbapenemase enzymes encoded by alleles of the bla_{KPC} gene is hydrolysed by the carbapenems antibiotic such as meropenem.[4,5] The plasmid-borne colistin resistance *mcr-1* was reported in *Escherichia coli* (*E. coli*) in the year 2015. Since then, colistin has become the last choice of drug to treat conditions caused by highly resistant pathogen.[6] Since 2015, eight additional *mcr* homologous (*mcr-1* to *mcr-9*) have been reported worldwide, and are chromosome mediated, among them *mcr-3*.[7–9] *Salmonella* species carrying *mcr-1* have been recovered from many specimens including food, animals and clinical in China. The occurrence and distribution of clinical *Salmonella enterica* serotype Typhimurium (*S*. Typhimurium) ST34 carrying *mcr-1* was found to be low so far.[10] Among the most recorded clinical isolates, producing *mcr*-1 recovered from bloodstream infection and a faecal sample is rare.[11] Young children are at high risk of acquiring *Salmonella* infection due to their underdeveloped immune system.[12] The increasing prevalence of MDR *Salmonella* in pediatric patients poses a serious challenge for treatment due to restricted drug of choice.[13] Here, we report a colistin-resistant *S*. Typhimurium ST34 with IncX4 plasmid carrying *mcr-1,* $bla_{\text{KPC-2}}$ and $bla_{\text{CTX-M-15}}$, which was isolated from a paediatric patient, suffered suffering from diarrhoea. Antibiogram, horizontal gene transformation and whole-genome sequencing were performed to demonstrate the molecular characteristics of the isolate.

Methods

Bacterial Isolation and Identification

Strain SP-15-127 was isolated from a faecal sample of a patient in 2015, in Shenzhen, China. Species primarily identification was done by using the VITEK 2 compact system (bioMérieux, France), followed by 16S rRNA Sanger sequencing from a commercial company (Sangon Biotech, Shanghai). The full length of 16S rRNA gene was amplified via conventional PCR by using F-5'-GGAACTGAGACACGGTCCAG −3' and R-5'-CCAGGTAAGGTTCTTCGCGT-3'. PCR reaction volume was 20 µL contained 1µL (30ng) of genomic DNA, 0.4 µL (10 pmol) of each forward and reverse primer, 10 µL of 2X Master Mix and 8.2 µL of nuclease-free water. Thermocycler set for 5 minutes at 95°C for initial denaturation, 35 cycles each 30 sec. 94°C for denaturation, 25 sec at 56°C for annealing and 50 sec at 72°C for extension and final extension at 72°C for 7 minutes, the PCR product was run on 1% agarose along with a DNA ladder. This isolate was collected as a routine Hospital investigation procedure. Only verbal consent was obtained because no personal information was used for research purposes, therefore written consent was not required. All experiments were conducted as per the hospital biosafety regulations act. The polymerase chain reaction (PCR) assay was performed to detect *mcr-1,* $bla_{\text{KPC-2}}$ and $bla_{\text{CTX-M-15}}$ using specific primers as we previously described.[14] Purified PCR products were sequenced (Sanger sequencing method) by Sangon Biotech-Shanghai, China. DNA sequences were analysed by the NCBI-BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was performed using VITEK 2 compact-60 system with ASTGN09 card and software version 9.01 (bioMérieux) for amikacin, aztreonam, nitrofurantoin, ciprofloxacin, piperacillin, gentamicin, cefepime, ceftriaxone, ceftazidime, tobramycin, imipenem, levofloxacin and sulfamethoxazole/trimethoprim as per manufacture instructions. The E-test method was used to determine the MIC value of meropenem, but for colistin, the broth dilution method was used in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. A positive control strain characterized from our laboratory was used, while ATCC25922 was used as a quality control strain.[4] Results were interpreted according to CLSI instructions, while colistin resistance was defined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints.[15,16]

Conjugation, CeuI-PFGE and Southern Hybridization

Conjugation was performed using the isolate SP-15-127 as a donor cell while streptomycin-resistant *Escherichia coli* strain C_{600} (*E. coli* C_{600}) was used as the recipient. The broth medium for both isolates were mixed and incubated for

meeting at 37° C for 24 hrs as described previously.[17] The conjugants were screened on Muller Hinton agar plates containing colistin (4 μg/mL) and streptomycin (2000 μg/mL) for 18 hrs. Thereafter, conjugants were selected for antibiotic susceptibility to determine the phenotypic expression of transferred genes followed by PCR assay and sequencing (Sanger sequencing). The plasmid and (or) chromosomal locations of *mcr-1* were determined by S1-PFGE, followed by southern hybridizations. The genomic DNA of the isolate SP-15-127 was digested with S1 endonuclease (Takara Biotech).[18] The digested DNA fragments were separated by CHEFDR III BioRad system with a run time of 12 hrs and switch time of 5–40 Seconds, and lambda ladder was used as a marker. Southern hybridizations of plasmid DNA were performed with a digoxigenin-labelled *mcr-1* probe, according to the manufacturer's instructions (Roche Diagnostics, Germany).

Whole-Genome Sequencing and Bioinformatic Analysis

The isolated *Salmonella enterica* serotype Typhimurium strain (SP-15-127) was subjected to whole-genome extraction using the Qiagen Blood & Tissue kit (Qiagen, Hilden, Germany, Lot No. 121223). DNA quantity and quality were analysed using gel electrophoresis and the BioDrop DUO UV/VIS spectrophotometer device (BioDrop England). The DNA library was prepared with 400bp paired-end fragment and sequencing was performed using an Illumina HiSeq 2000 platform. The mixed assembly of Illumina was assembled into contigs using SPAdes version 3.11.1 and the quality of the assemblies was evaluated using the software QUAST. Plasmid incompatibility, multi-locus sequences typing (MLST), antimicrobial resistance genes (AMR) and virulence genes were identified using the Centre for Genomic Epidemiology (CGE) platform (http://www.genomicepidemiology.org/services/).[19]

Results

Isolate SP-15-127 Characteristics

Isolate SP-15-127 was conferred as *S.* Typhimurium which was recovered from a faecal sample of a 6-year male child diagnosed with gastroenteric and bacteraemia. The PCR assay demonstrated that SP-15-127 harbouring colistin resistance *mcr-1*, carbapenemase encoding $bla_{\text{KPC-2}}$ and extended β-lactamase encoding $bla_{\text{CTX-M-15}}$ genes which hydrolysed the ceftazidime and ceftriaxone. Antibiogram results indicated that isolate SP-15-127 was resistant to most antibiotics including colistin, aztreonam, nitrofurantoin, ciprofloxacin, piperacillin, cefepime, ceftriaxone, ceftazidime, levofloxacin, and imipenem but sensitive to the tobramycin, tigecycline (Table 1).

Transferability and Location of Genes

Performing the conjugation experiments, we found that isolate was able to transfer their colistin resistance phenotype to *E. coli* C_{600}. The frequency of conjugation for the *mcr-1* gene was 5.6% in isolates. S1-PFGE and Southern hybridization indicated that *mcr-1* gene was located on a 40kb plasmid belonging to IncX4 (Figure 1). The resistant phenotype showed that conjugants were resistant to aztreonam, piperacillin, cefepime, ceftriaxone, ceftazidime, and imipenem (Table 1). The PCR product sequencing revealed that both $bla_{\text{KPC-2}}$ and $bla_{\text{CTX-M-15}}$ was also located on the same plasmid.

Table 1 Antimicrobial Susceptibility of *Salmonella* Sp-15-127, Transconjugant's and *E. Coli* C_{600} (Recipient)

Isolates	MIC (mg/mL)														
	AMK	ATM	FT	CT	CIP	CRO	TZP	GEN	FEP	CRO	CAZ	TM	IPM	LEV	SXT
Salmonella sp-15-127	16	≤1	128	8	≥4	≥64	≥128	≥32	≤1	≥64	≥64	≥16	16	≥16	≤20
ATCC25922	≤2	≤2	≤1	≤0.25	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Transconjugant's	≤2	≤2	16	4	2	≥64	64	≤16	≤1	≥64	≥64	≥16	8	≤1	≤20
E. coli C_{600} (recipient)	≤2	≤2	≤1	≤0.25	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤20

Abbreviations: AMK, amikacin; ATM, aztreonam; FT, nitrofurantoin; CT, colistin; CIP, ciprofloxacin; CRO, ceftriaxone; TZP, piperacillin; GEN, gentamycin; FEP, cefepime; CRO, ceftriaxone; CAZ, ceftazidime; TM, tobramycin; IPM, imipenem; LEV, levofloxacin; SXT, trimethoprim/sulfamethoxazole.

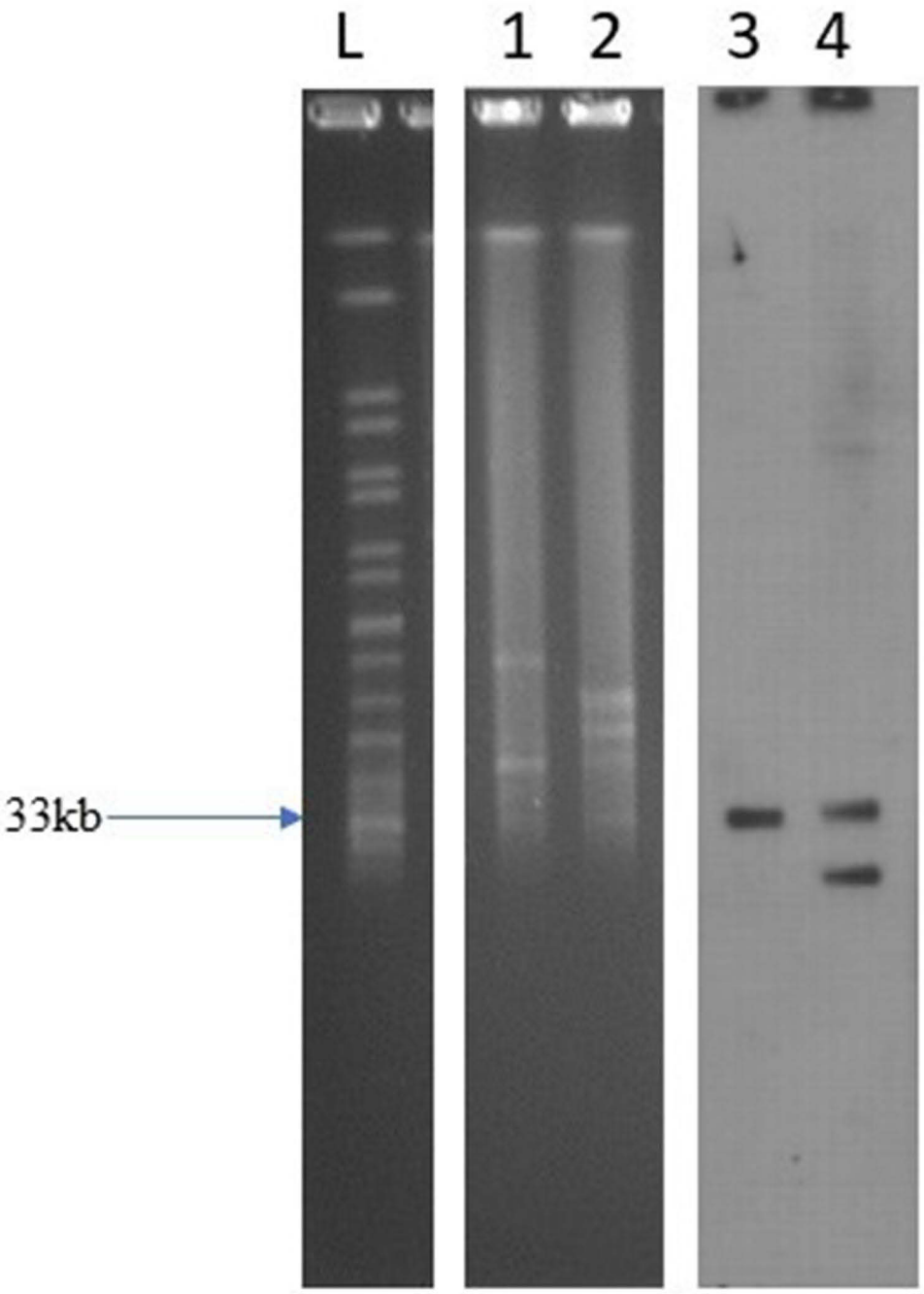

Figure 1 S1-PFGE pattern for SP-15-127 strain and Southern blot analysis of *mcr-1* genes.
Notes: Lane-L: Lamba marker, Lane 1: PFGE result of I-CeuI-digested DNA of SP-15-127; Lane 2: PFGE patterns for I-CeuI-digested DNA of the transconjugants C_{600}; Lane 3 and 4: Southern hybridization with the probes specific to the *mcr-1* gene.

Genome Characterization

MLST results revealed that *S*. Typhimurium belongs to the ST34 group. The isolate of a single plasmid belonging to the Incx4 was confirmed using Plasmid Finder. The genetic context of the MCR-1, CTX-M-15 and KPC-2 in the plasmid was represented in (Supplementary Material 1). Our genomic characterization data reports the plasmid-encoded resistance genes, such as colistin aminoglycoside [*aadA1* and *aac(3)-IVa*], trimethoprim (*dfrA12*), sulphonamide (*sul1, sul2 and sul3*), phenicol (*floR* and *cmlA1*) and fosfomycin (*fosA3*). We also found evidence of IncX3 plasmid harbouring the $bla_{\text{TEM-1}}$ (cephalosporin resistance), *floR* (florfenicol resistance), *tet(*A) (tigecycline resistance) and $bla_{\text{SHV-12}}$

(carbapenem resistance) genes. Most of the resistance genes are located on the plasmids, indicating transferability and transmission of the corresponding resistance genes to other bacteria. Copper resistance gene operons (cus operon and cop operon) and silver resistance genes *(silP and silE)* were reported, which was found to be responsible for the heavy metal resistance in this isolate.

Discussion

The prevalence of colistin-resistant bacteria is increasing attention, therefore considered as a threat to global health.[20] Colistin is the last choice of drug to treat conditions caused by carbapenemase-producing bacteria.[21] Nevertheless, we found the emergence of the plasmid-borne colistin resistance gene *mcr-1* co-existence and co-transmission with extended beta-lactamase encoding genes as well as carbapenemase encoding genes such as bla_{KPC}, and $bla_{\text{CTX-M}}$, which are located on transferable element plasmid.[22,23] The prevalence of *mcr-1* harbouring *S*. Typhimurium is highly in animal husbandry but still low in humans, specifically in children.[24,25] The *K. pneumoniae* highest *mcr-1*-positive rate is about 10%, which is quite higher than *salmonella* in China.[26] Furthermore, the co-existence of *mcr-1* (bla_{KPC} and $bla_{\text{CTX-M}}$) in one *S*. Typhimurium strain is not yet reported in China. In this study, only one isolate among nearly thousands of clinical *S*. Typhimurium strains was confirmed as a multidrug-resistant superbug that harboured these three important resistance genes. Unfortunately, the *mcr-1,* $bla_{\text{KPC-1}}$ and $bla_{\text{CTX-M-15}}$ genes were located on a single plasmid, which has a high possibility of their co-transfer. The multidrug-resistant strain SP-15-127 belongs to ST34 based on the *S*. Typhimurium MLST scheme. Previous studies on *S*. Typhimurium ST34 were associated with copper resistance and *toxin* production in the gut.[27,28] In addition, ST34 strains were found to be a dominant host for the mcr-1-IncX4 plasmid, which possessed a highly conserved sequence and plasmid structure. Moreover, the carbapenemase gene bla_{NDM}, and $bla_{\text{CTX-M}}$ has been reported occasionally, but bla_{KPC} emerged rarely in the ST34 strain.[29] Plasmids play a key role in harbouring and transferring resistance genes, especially in *Enterobacteriaceae*.[4] We harvested single plasmid IncX4 from SP-15-127 strain, which encode resistance genes including *mcr-1,* $bla_{\text{KPC-2}}$, and $bla_{\text{CTX-M-15}}$. Luo et al have reported the presence of *S*. Typhimurium ST34 carrying mcr-1 in a paediatric patient with bloodstream infection, and the genomic location was pHNSHP45-2-like IncHI2 plasmid.[9] In this study, the mcr-1 harbouring plasmid IncX4 was highly distributed in *E. coli*, suggesting that the transmission of plasmid may occur from *E. coli* to *Salmonella*. The *mcr-1* location on IncX4 plasmid is considered a major reservoir and is highly disseminated in China, both in humans and the environment.[30] Our reported plasmid has a high transfer rate (5.6%) which was similar to the one reported by Jian et al. In their study, they screened IncX4 plasmids among 2470 isolates of *Enterobacteriaceae* and determined the transfer rate.[31] One of the potential limitations of this study is the whole plasmid sequence.

Conclusion

Here, we report the occurrence of ST34 of *Salmonella enterica* serotype Typhimurium carrying multiple drug resistance encoding genes co-harbouring *mcr-1,* $bla_{\text{KPC-2}}$ and $bla_{\text{CTX-M-15}}$ in IncX4 plasmid. These resistant determinants present on the mobile element of plasmid, potentially, transmit genes via horizontal gene transfer and were responsible for the emergence of the superbug. Our findings also suggest the need for epidemiological surveillance and monitoring of *Salmonella* superbug transmission emergence.

Data Sharing Statement

All data files mentioned in this manuscript are available.

Ethics Approval and Consent to Participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee, Shenzhen Children's Hospital, Reference number: 2018 (013) on dated 2018/09/03.

Informed Consent Statement

Due to the retrospective nature of the study, the Ethics Committee of Shenzhen Children's Hospital, Shenzhen determined that patients' consent was not required. The clinical isolate samples used in this research were part of the

routine hospital laboratory procedure No personal patient's information was used, data were kept confidentially and in compliance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and also agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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