Plasmid-Encoded *bla*_{NDM-5} Gene That Confers High-Level Carbapenem Resistance in *Salmonella* Typhimurium of Pork Origin

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Yuan Gao,¹ Junping Wen,¹ Shaojun Wang,¹ Xuebin Xu,² Zeqiang Zhan,¹ Zhengquan Chen,¹ Jie Bai,¹ Xiaoyun Qu,¹ Hongxia Zhang,¹ Jianmin Zhang,¹ Ming Liao¹

¹National and Regional Joint Engineering Laboratory for Medicament of Zoonoses Prevention and Control, Guangdong Laboratory for Lingnan Modern Agriculture, Key Laboratory of Zoonoses, Ministry of Agriculture, Key Laboratory of Animal Vaccine Development, Key Laboratory of Zoonoses Prevention and Control of Guangdong Province, College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong, People's Republic of China; ²Department of Microbiology, Shanghai Municipal Centre for Disease Control and Prevention, Shanghai, People's Republic of China

Correspondence: Ming Liao; Jianmin Zhang
National and Regional Joint Engineering Laboratory for Medicament of Zoonoses Prevention and Control, Guangdong Laboratory for Lingnan Modern Agriculture, Key Laboratory of Zoonoses, Key Laboratory of Animal Vaccine Development, Ministry of Agriculture, Key Laboratory of Zoonoses Prevention and Control of Guangdong Province, College of Veterinary Medicine, South China Agricultural University, No. 483 Wushan Road, Tianhe District, Guangzhou 510642, Guangdong, People's Republic of China
Tel +86 020 85290240;
+86 020 85280240
Fax +86 020 85290240;
+86 020 85285282
Email mliao@scau.edu.cn;
junfeng-v@163.com

Purpose: Carbapenem resistance is rarely reported in *Salmonella* Typhimurium, especially from a food origin. Here, we report a plasmid-mediated mobile carbapenem-resistant *bla*_{NDM-5} gene in *Salmonella* Typhimurium isolated from pork in Shanghai, China in 2016.

Patients and Methods: In July 2016, the *S. Typhimurium* SH160 strain was recovered from minced pork meat purchased from a supermarket in Yangpu District, Shanghai, China. Antimicrobial susceptibility testing, multi-locus sequence typing, conjugation, S1-PFGE, southern hybridization, whole-genome sequencing and data analysis were performed.

Results: This isolate was found to be a ST34 strain and resistant to carbapenems, cephalosporins, and most other commonly used antibiotics. The *bla*_{NDM-5} gene was harbored by a 46161-bp IncX3 plasmid which was found to be transferable. The IncX3 plasmid contains a composite cassette, consisting of *ISSw1-IS3000-AISAb125-IS5-bla*_{NDM-5}-*bleMBL-trpF-dsbC-IS26-ctuA1-AumuD*. In addition, this strain was found to harbor an additional 161706-bp IncHI2 plasmid which carries nine resistant genes, such as *aadA1*, *aadA3*, *aph(3')-Ia*, *sul1*, *sul2*, *sul3*, *floR*, *cmlA* and *dfrA12*.

Conclusion: We reported the *S. Typhimurium* with transferable IncX3 plasmid harboring *bla*_{NDM-5} gene from minced pork. We characterized the complete genetic features of the plasmid, which demonstrated the potential for spreading in different bacterial pathogens. Therefore, extensive surveillance and monitoring for carbapenem-resistant bacterium in the food chain and public health are urgently required.

Keywords: *S. Typhimurium*, pork, *bla*_{NDM-5}, public health

Introduction

Salmonella is an important foodborne pathogenic bacterium that causes considerable morbidity and mortality. The burden of foodborne non-typhoidal serovars of *Salmonella enterica* (NTS) infections is extremely high, with a global estimation of 78.4 million cases, 28,693 deaths, and over 2 million disability adjusted life years (DALYs) in 2010.¹ The emergence and spread of multidrug resistance (MDR) have occurred in *Salmonella enterica* serovar Typhimurium strains, which has had a significant impact on the effectiveness of current strategies to control and manage diseases associated with foodborne infections.^{1,2}

The trend of MDR in *Salmonella* has increased each year in China.³ Of particular concern is the emerging resistance to carbapenems, since these agents are often regarded as the 'last-line' of effective therapy for treating infections caused by MDR Gram-negative bacteria.⁴ Moreover, there has been an increase in the spread of

carbapenemase-encoding genes among nosocomial enteric bacteria, particularly *Klebsiella pneumoniae* and *Escherichia coli*.⁵ Although carbapenemase-producing bacteria have been reported in clinical infections, there are sporadic reports of *Salmonella*, especially those involving a food origin. Due to the emergence of such resistance in foodborne pathogens, active surveillance and monitoring for carbapenem-resistant bacteria in the food chain is urgently required, as they may be transferred to both humans and the environment.⁶

Therefore, this study aims to describe the occurrence of carbapenemase-carrying and MDR *Salmonella* Typhimurium isolated from retail pork in Shanghai and investigate the mechanism of the plasmid-mediated carbapenem-resistant *Salmonella enterica* serovar. Whole-genome-sequencing (WGS) was used to determine the complete plasmid profile, followed by a comparative genetic analysis, to lay the foundation for follow-up studies related to food safety and public health issues caused by *Salmonella*.

Materials and Methods

Specimen Collection, Isolate Identification and Multilocus Sequence Typing (MLST)

In July 2016, the *S. Typhimurium* SH160 strain was recovered from minced pork meat in a supermarket in Yangpu District, Shanghai, China. *Salmonella* was isolated according to the US FDA Bacteriological Analytical Manual.⁷ Isolates with typical *Salmonella* phenotypes were further confirmed using API identification kits (bioMérieux, France). The O and H antigens were characterized using slide agglutination with *Salmonella* diagnostic serum (S&A Reagents Lab, Bangkok, Thailand). The serological determination of *Salmonella* serotypes was performed in accordance with the Kauffmann-White scheme.⁸ Multi-locus sequence typing (MLST) was performed using primer sets ([Supplemental Table 1](#)). Sequences of seven housekeeping genes (*aroC*, *dnalN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were compared with the available MLST database (<http://mlst.warwick.ac.uk/mlst/dbs/Senterica>) to get the allele number and sequence typing (ST) number for each isolate. Sequence information for newly assigned alleles and STs was deposited in the MLST database.

Antimicrobial Susceptibility Testing and Identification of the bla_{NDM-5} Strain

The susceptibility of *S. Typhimurium* SH160 strain to different antibiotics was determined using the standard agar dilution method and interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) standard.⁹ *E. coli* strain ATCC25922 was used as a quality control strain. The PCR amplification of the *bla*_{NDM-5} gene was performed to identify the presence of *bla*_{NDM-5} gene (Primers are in the [Supplemental Table 2](#)).

Conjugation, SI-PFGE, and Southern Hybridization Assay

To test the transferability of the *bla*_{NDM-5}-bearing plasmid, conjugation by filter mating was performed between *S. Typhimurium* SH160 strain and streptomycin-resistant *E. coli* EC600. Both the donor and recipient strains were cultured to the exponential phase in LB broth. The donor and recipient bacteria were mixed at a 1:4 ratio and incubated overnight at 37°C. The mixture was then spread onto a selective MacConkey agar plate containing imipenem (2 mg/L), and streptomycin (3000 mg/L) to select for transconjugants that had acquired the *bla*_{NDM-5} bearing plasmid. To estimate the size and location of *bla*_{NDM-5} in the parental strain and the transconjugants, DNA linearization with S1 nuclease followed by PFGE (S1-PFGE) and Southern hybridization analyses were performed. Southern hybridization was performed in accordance with the manufacturer's instructions of the digoxigenin (DIG)-High Prime DNA Labelling and Detection Starter Kit II (Roche Diagnostics) using *bla*_{NDM-5} digoxigenin-labelled probes (primers for the probe are in the [Supplemental Table 2](#)).

Whole-Genome Sequencing and Comparison Analysis

The whole *S. Typhimurium* SH160 genome was sequenced using PacBio RS II Single Molecule Real-Time (SMRT) and Illumina sequencing platforms. The raw reads were then assembled into a contig using the hierarchical genome assembly process (HGAP) and Canu.^{10,11} The complete plasmid sequence was predicted with GLIMMER and annotated with the NR, Swiss-Prot, Pfam, GO, COG, and KEGG databases using sequence alignment tools, including BLAST, Diamond and HMMER.^{12–15} Easyfig was used in comparative analysis (Genbank accession number KY435936, KF220657, KF8

77335).^{16,17} ResFinder and PlasmidFinder were used to detect drug-resistant genes and the plasmid sequencing type.^{18,19}

Results

S. Typhimurium Isolation and Antimicrobial Susceptibility Testing

Multi-locus sequence typing analysis revealed that SH160 belonged to sequence-type 34 (ST34). The *S. Typhimurium* strain, SH160, was found to be resistant to a wide range of antibiotics, including imipenem, ampicillin, cefotaxime, cefepime, gentamycin, nalidixic acid, ofloxacin, florfenicol, and sulfisoxazole (Table 1). However, the strain was susceptible to amikacin, ciprofloxacin tetracycline, streptomycin, and polymyxin B.

Conjugation, S1-PFGE, and Southern Hybridization

The *bla_{NDM-5}* gene was successfully transferred to *E. coli* EC600. The transconjugant was resistant to cephalosporin, imipenem, ampicillin, gentamycin, florfenicol, and sulfisoxazole. S1-PFGE and Southern hybridization indicated that the *bla_{NDM-5}* gene was located on a conjugative plasmid, with a size of approximately 46 kb, designated pNDM5_SH160 (Figure 1).

Whole-Genome Sequencing and Comparison Analysis

A 46161-bp *bla_{NDM-5}*-carrying IncX3 plasmid, pNDM5_SH160, and a 161706-bp IncHI2 plasmid were identified in SH160, which exhibited G+C content of 46.74% and 46.48%, respectively. These plasmids also contained 62 and 163 predicted coding sequences (CDSs), respectively. The *bla_{NDM-5}* gene is adjacent to an incomplete *ISAbal25*, which was interrupted by the insertion of *IS5* and truncated by an insertion of *IS3000* at its left end. A zinc metalloproteinase-encoding gene, *mpr*, lies further upstream of *bla_{NDM-5}*. Several putative open reading frames (ORFs) of an unknown function and a truncated *ISSwil* was found to be present between *mpr* and *IS3000*. There are *bleMBL* (mediating bleomycin resistance), *trpF* (encoding a phosphoribosyl anthranilate isomerase), *dsbC* (encoding an oxidoreductase), a remnant of *ctuA1* (encoding an iron-tolerant protein), and a truncated *umuD* gene (encoding a mutagenesis protein) nearby (Figure 2). In addition to the *bla_{NDM-5}* gene, multiple resistance genes were identified, including *aadA1*, *aadA3*, *aph(3')-la*, *aph(4')-la*, *sul1*, *sul2*, *sul3*, *floR*, *cmlA*, and *dfrA12*, which were all located in the IncHI2 plasmid (Table 1).

Table 1 The Minimum Inhibitory Concentration (MIC) of High-Resistant *Salmonella* Typhimurium SH160

Antibiotic Category	Antibiotics	MIC ^a (μg/mL)	Transconjugant's MIC (μg/mL)	MIC Standard ^b (μg/mL)			Plasmid Carrying Resistant Gene
				Sensitive (S)	Intermediate (I)	Resistant (R)	
Penicillin	Ampicillin	>512	256	≅8	16	≅32	
Cephalosporin	Cefotaxime	32	32	≅	2	≅4	<i>bla_{NDM-5}</i>
	Cefepime	16	2	≅2	–	≅16	
Aminoglycosides	Gentamicin	128	8	≅4	8	≅16	<i>aac(3)-IV</i> , <i>aadA1</i> , <i>aadA3</i> , <i>aph(3')-la</i> , <i>aph(4')-la</i> ,
	Streptomycin	16	>1024	–	–	≅64	
	Amikacin	8	1	≅16	32	≅64	
Quinolones	Ofloxacin	1	0.125	≅2	4	≅8	
	Ciprofloxacin	2	0.25	≅0.06	0.12–0.5	≅1	
Sulfonamides	Nalidixic acid	>512	64	≅16	–	≅32	<i>sul1</i> , <i>sul2</i> , <i>sul3</i> , <i>dfrA12</i>
	Sulfisoxazole	>512	64	≅256	–	≅512	
Chloramphenicols	Florfenicol	64	64	≅4	8	≅16	<i>floR</i> , <i>cmlA</i>
	Chloromycetin	128	4	≅8	16	≅32	
Colistin	Polymyxins B	0.5	0.125	≅2	4	≅8	
Tetracyclines	Tetracycline	4	4	≅4	8	≅16	
Carbapenems	Imipenem	8	4	≅1	2	≅4	<i>bla_{NDM-5}</i>

Notes: ^aThe MIC of *Salmonella* Typhimurium of SH160. ^bAll MICs were interpreted according to Clinical and Laboratory Standards Institute (CLSI).

Figure 1 S1-PFGE and hybridization indicating the transfer of single plasmid harbouring bla_{NDM-5}. Lane 1, M, H-9812 (PFGE marker strain, *S. Braenderup* H9812; Lane 2, SH160, *S. Typhimurium* SH160 (donor); Lane 3, Tr160, EC600 (transconjugant)).

The results showed that the bla_{NDM-5}-bearing plasmid shared 100% query coverage with several other bla_{NDM-5}-harbouring plasmids, as well as >99% sequence identity with the plasmid, pNDM_MGR194, which was isolated in India.²⁰ The genetic background of bla_{NDM-5} in these two plasmids is identical (*ISSwil-IS3000-ΔISAb125-IS5-bla_{NDM-5}-bleMBL-trpF-dsbC-IS26-ctuA1-ΔumuD*). Such a background of the bla_{NDM-5} gene in IncX3 plasmids is highly similar to that of bla_{NDM-1} and bla_{NDM-5} in several other IncX3 plasmids (Figure 2).

Discussion

Carbapenems are critical 'last-resort' antibiotics reserved for the treatment of serious infections caused by certain highly

resistant Gram-negative bacteria. In contrast to other *Enterobacteriaceae* (eg, *K. pneumoniae*, *E. coli*, and *Enterobacter* spp.), carbapenem-resistant *Salmonella* has rarely been reported.^{21–23} We report the successful isolation and characterization of a metallo-beta-lactamase (MβL) encoding bla_{NDM-5} gene harboured in *S. Typhimurium* isolated from pork meat in Shanghai, China. This strain together with the previous isolation from Jiangsu province confirms the prevalence of bla_{NDM-5} in food chains.²⁴ Therefore, there is a potential risk of cross-contamination in food, animals, and humans. Hence, identifying the mechanism of the spread of carbapenem-resistant *Salmonella* in the environment has become a substantial global health concern.

According to the MLST results, the SH160 strain belongs to ST34, which is one of the most prevalent types of *S. Typhimurium*. Moreover, ST34 clones have raised international concern regarding their rapid prevalence and high level of drug resistance. A previous study demonstrated that *S. Typhimurium* ST34 clones experienced a rapid expansion in China and exhibited resistance to cephalosporin antibiotics.²⁵ Notably, *S. Typhimurium* ST34 SH160 strain is not only resistant to several common antibiotics due to harbouring of a variety of resistant genes in the IncHI2 plasmid, but also resistant to carbapenem and cephalosporins due to the bla_{NDM-5} gene in IncX3 plasmid. Therefore, the surveillance of *S. Typhimurium* ST34 should be strengthened to reduce the probability of drug-resistant plasmid transmission.

In this study, we identified the pNDM5_SH160 and the IncHI2 plasmids were transferable indicating the potential risk of horizontal transmission of drug resistance. In addition, the comparison of pNDM5_SH160 showed that *E. coli*, *Klebsiella pneumoniae*, and *Raoultella planticola* share a mostly conserved plasmid backbone (*ISSwil-IS3000-ΔISAb125-IS5-bla_{NDM-1/5}-bleMBL-trpF-dsbC-IS26-ctuA1-ΔumuD*), including the bla_{NDM-5}/bla_{NDM-1} gene, which shows the prevalence of the plasmid with a strong transmissibility among different species widely (Figure 2). On the other hand, IncX-type plasmids are narrow-host range plasmids of *Enterobacteriaceae* and include at least five subtypes: IncX1–IncX5. In addition, some IncX3 plasmids carrying ESBL and/or the carbapenemase gene from various species of *Enterobacteriaceae* found in several countries have been completely sequenced.²⁶

Since the bla_{NDM-5} gene was first identified from *E. coli* in the UK,²⁶ strains containing the bla_{NDM-5} have emerged in several countries.^{20,27} Nevertheless, the bla_{NDM-5} gene has not been identified in *Salmonella enterica* of pork origin in

Figure 2 Features of the genetic structure of pNDM5_WCHEC0215 (Genbank accession number KY435936), pNDM_MGR194 (Genbank accession number KF220657), pNDM5_SH160, and RJA274 plasmid (Genbank accession number KF877335). The bla_{NDM-1/5} gene is shown in red. IS3000, ISAbal25, and other insert sequences are shown in green. The replicons are shown in blue. The rest of the genes are shown in orange.

Shanghai.⁶ NDM-5 differs from NDM-1 by two amino acid substitutions (Val88Leu and Met154Leu) and confers high-level resistance to carbapenems and broad-spectrum cephalosporins.²⁸ As a result, plasmids carrying NDM variants might be widely disseminated, posing a potential public health threat.

Conclusion

We reported the carbapenem-resistant *Salmonella* Typhimurium from minced pork and identified that carbapenem-resistance was mediated by a transferable plasmid. Complete genetic features of pNDM5_SH160 and comparison were performed. Such emergence of NDM carrier plasmids indicates that we should develop strategies to control the spread. Extensive surveillance and monitoring of the carbapenem-resistant bacterium in the food chain are urgently required.

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Disclosure

The authors report no conflicts of interest in this work.

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